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Trends in Cancer Research[1]

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On behalf of the National Cancer Institute, I wish to acknowledge our indebtedness to your college, first, for improving the standards of tumor clinics, which has been of great value to us in the effective distribution of radium loans and in the provision of free training for physicians in diagnosis and treatment,[2] and second, for having kept alive the constructive interest of the medical profession and laity in the cancer problem.

Trends in any field of research change with time as new facts are discovered. Up to the end of the last century cancer research was confined to descriptive observations on cancer in man. Isolated reports of cancer in other mammals were ignored. Speculation was rampant as to causation. A remarkable exception was the keen observation of Percival Pott, who, in 1775, recognized the possible relationship of coal soot and cancer of the scrotum in chimney sweeps. A century later, Virchow's hypothesis focused attention on the tissue cells, which were supposed to undergo malignant transformation as a result of "chronic irritation." Subsequent modifications of the cell theory attributed cancer to embryonic cell rests or to abnormal conditions during regeneration of injured tissues. With the recognition of the parasitic origin of certain diseases, the search for a specific infectious agent for cancer was let loose and to some extent has continued to this day. In retrospect it is clear that each of these attempts to explain the causation of cancer had the weakness of oversimplifying the problem. At last, at the end of the nineteenth century, Claude Bernard's plea for controlled experimentation was applied to the cancer problem. As a result a large number of new facts have been discovered. In order to limit the scope it is necessary to restrict the following discussion to mammalian neoplasms.

The first important conclusion reached from experimental work is the high specificity of malignant cells. Each one of the the many types breeds true *in vivo* and *in vitro*. For instance, a mammary carcinoma of the mouse transplanted into other susceptible mice reproduces itself in an unlimited series of generations. Furthermore, many types of malignant cells have been maintained in tissue culture for years without losing the ability to produce the specific tumors after being inoculated into the same strain of animals from which they were derived. The maintenance of

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such cultures over a long time requires an aseptic technique which is more strict than that used in surgery. The slightest infection as a rule causes the loss of cultures. It is difficult to bring these and other facts into harmony with the view that mammalian cancers are due to a specific infectious agent, especially since it has not been possible to isolate such agents from any tumors. Moreover, numerous attempts to produce curative serums without exception have failed. On the other hand it is established, both clinically and experimentally, that certain parasitic infections can predispose tissues to the development of cancer, but it should again be emphasized that these parasites do not play a specific role and are not detectable in the resulting neoplasms.

Basing their investigation on Pott's observation, Yamagiwa and Ichikawa in 1915 announced the production of skin cancer in rabbits by the long-continued topical application of coal tar. This important discovery shifted the trend of cancer research into the field of chemistry. Through painstaking work a highly potent carcinogenic chemical, 3,4-benzpyrene, was isolated from coal tar. At present the carcinogenic properties of about 250 synthetic chemicals have been recognized. Some of these, as 20-methylcholanthrene, have been extremely valuable for the experimental production in animals of almost every type of malignant tumor seen in man. Fractions of a milligram of this compound can produce malignant tumors at the site of injection or in distant organs.

The ease of chemical carcinogenesis has thrown much light on tumor histogenesis under controlled conditions (*3*, *6*, *7*). Morphologically recognizable tissue injury may occur but does not necessarily precede tumor formation. As exceptions, I mention induced as well as spontaneous pulmonary and mammary tumors. In fact, the term "chronic irritation," while still of value for cancer-control education, has outlived much of its scientific usefulness in cancer research. The conversion of normal into malignant cells is apparently due to changes which are far too subtle to be analyzed exhaustively by the available histologic methods. Even the most refined cytologic technique at present fails to differentiate malignant cells from the cells of origin. After all, what we see through the microscope is only an infinitesimal part of what is present and going on in living cells. For this reason I have maintained for some years that carcinogenesis, as well as other fundamental cancer problems, should be attacked from the viewpoint of cell physiology and biochemistry, for we are dealing here first and last with a fundamental biologic problem.

Work with chemical carcinogens has firmly established the fact that tumors appear only after a relatively long latent period, which depends on the type, dose, and method of administration of the chemical, the animal species and strain, and the sex (*2*). The great value of animals with a relatively short life span of 2 to 3 years, such as mice and rats, may well be due in part to the rapidity of aging, which may be assumed also to speed up the cancer process. The physiologic time factor is certainly important in human and animal carcinogenesis. Therefore, a better understanding of carcinogenesis will go hand in hand with a better understanding of the process of aging, of which we know relatively little.

Another remarkable fact is the ease with which such a powerful chemical as methylcholanthrene can produce malignancies in a great variety of tissues, as the brain, skin, striated muscle, subcutaneous tissue, lung, et cetera. This lack of specificity with regard to the cell type involved deserves further study. It is, indeed, exceedingly im-

portant to explain the intricate mechanism of action of methylcholanthrene on any normal cell type which is known to be convertible into the malignant state. Work on this subject has been in progress for some time at the National Cancer Institute and elsewhere. We have found, for instance, that prolonged exposure of cultures of normal subcutaneous mouse fibroblasts to high dilutions of methylcholanthrene causes apparently permanent changes in cell physiology, comparable with those observed in cultures of sarcomas induced by methylcholanthrene in the subcutaneous tissue of mice of the same strain (*4*). Efforts are being continued to prove that carcinogenesis can take place *in vitro*. Positive results would be of great theoretical interest, since this would indicate that carcinogenesis does not depend on favorable systemic conditions, but can be explained by a direct interaction between carcinogen and cell.

Recently some progress has been made in the study of the dietary factor in the production of induced tumors. Tissue cells are highly organized biochemical units. Their normal function depends partly on a continuous supply of energy-yielding chemicals and of other chemicals which are needed for the construction of cell proteins, lipids, and carbohydrates, or for the regulation of cell activity, as enzymes and vitamins. Hence it is logical to consider the composition of the diet as a possible modifying factor in carcinogenesis. Some years ago, we (*19*, *20*) showed that the growth rate of spontaneous mammary carcinomas in mice could be strikingly inhibited by diets deficient in certain essential amino acids. This inhibition of tumor growth was not accompanied by any evidence of general malnutrition. Recently other workers found that the development of hepatomas in rats induced by the azo dye, butter yellow, can be considerably delayed by certain diets (*16*). Work at our Institute (*23*) has also shown that the response of mice to skin painting with methylcholanthrene is strikingly influenced by the composition of the diet. If the diet contains an adequate amount of the amino acid, cystine, the mice develop acute leukemia with great regularity. On a low cystine diet, however, the development of leukemia is greatly retarded, and instead the mice exhibit extensive sclerotic lesions of the larger arteries.

Butter yellow and methylcholanthrene are toxic for young rats maintained on a diet low in cystine. The growth rate is strikingly inhibited and is accelerated as soon as cystine is added to the diet (*22*, *24*). Dietary cystine may be regarded as a detoxicating agent for the two chemical carcinogens in question. Hepatoma formation by butter yellow is accompanied by severe liver injury and liver cirrhosis, which may or may not be a requisite for hepatoma formation (*5*). It is not easy, therefore, to differentiate the specific carcinogenic action from the nonspecific toxicity. Similar work on spontaneous tumors has also yielded results indicating a dietary influence.[2a] This promising field deserves further investigation.

It has been suggested that abnormal diets may play a part in the causation of gastrointestinal tumors, an idea which at least has the merit of stimulating research. Roffo (*14*) reported that rats which are maintained for a long time on a diet containing boiled fats develop gastric adenocarcinoma. Careful work at our Institute so far has not confirmed this claim. On

[2a] *Doctors White and Andervont, of this Institute, recently made the following observations. Virgin C3H mice when 4 weeks of age were divided into two groups. One group was fed a relatively low protein-low cystine diet and the other a relatively low protein-high cystine diet. At the end of 10 months all mice on the high cystine diet had developed spontaneous mammary tumors, whereas at 15 months none of the mice on the low cystine diet had mammary tumors.*

the other hand, we have succeeded in producing for the first time gastrointestinal carcinoma in mice by means of methylcholanthrene (*15*). The forestomach of the mouse is lined by squamous epithelium and the rest of the organ by glandular mucosa. Spontaneous gastrointestinal tumors have never been observed in the mice used. At first the carcinogen was given continuously to the mice as a dilute emulsion in the drinking water. After many months there occurred squamous-cell carcinomas in the forestomach, whereas the glandular portion was normal. Some of the animals also developed typical carcinoma of the small intestine (*13*). This curious susceptibility to malignant change of gastric squamous epithelium and the apparent lack of susceptibility of the gastric glandular mucosa must be explained. Can it be due to the inability of the carcinogen to penetrate into the gastric mucosa? To test this, a small volume of a dispersion of methylcholanthrene was injected into the glandular mucosa of mice of the same strains. Up to the present five typical adenocarcinomas have thus been produced at the site of injection. One of these tumors has been successfully transplanted subcutaneously into other mice. The transplants grow rapidly, and the tumors have retained their histologic structure in two generations. To me the most interesting point is the probability that the layer of gastric mucus functions as a protective barrier when the carcinogen is given orally. If this be true, it follows that the production of gastric adenocarcinoma by carcinogenic dietary components, if such exist, may be conditioned by a break in the mucus barrier under pathologic conditions.

The discovery of the artificial chemical carcinogens is a milestone in cancer research as it has made possible the experimental production of tumors under controlled conditions. Nevertheless, the great unsolved problem still is the causation of so-called spontaneous neoplasms. The ease with which the artificial agents produce tumors strengthens the belief that the naturally occurring tumors are caused by chemicals formed within the body. In fact, it is firmly established that very large doses of female sex hormones acting over a long time can induce malignant tumors of the breast and uterus in mice and rats. Furthermore, experiments with different inbred strains of mice have revealed a unique influence of the mother's milk on the development of mammary cancer in the offspring (*1*). Foster nursing of newly born female mice from a strain with a normally high tumor incidence by a mother of a low tumor strain strikingly decreases the tumor incidence in the foster-nursed animals. On the other hand, foster nursing of young females from a strain with a normally low tumor incidence by a mother of a high tumor strain greatly increases the tumor incidence in the fostered mice. Work on the nature of the agents responsible for these effects is in progress at our Institute, as is also the question as to whether or not the findings apply to human mammary carcinoma.

Mention may now be made of recent claims of the carcinogenic action of cell-free extracts of tissues and of urine from cancer patients. This work is based on the assumption that as a consequence of abnormal metabolism endogenic substances exert a carcinogenic action. The first report in this field was made by the Russian scientist, Schabad (*12*). He extracted grossly normal livers of individuals dead from cancer of the stomach, lung, and other organs, with benzene, since benzene is a good solvent for many carcinogenic hydrocarbons, sterols, and bile acids. After evaporation of the benzene extract the residue was injected subcutaneously into mice. This caused local tissue destruction

and killed some of the animals. Malignant tumors were observed after 8 months or longer, both at the injection site and in distant organs. Few tumors also occurred in nontreated control mice. Similar results have been reported by British and American investigators. Taken as a whole, these findings should be accepted with considerable caution. While there is no doubt that malignant tumors were observed, it is premature to accept this evidence as conclusive proof for the existence of endogenic carcinogenic chemicals in cancer patients. Most of the tumors appeared after a very long latent period and in a relatively low percentage of injected animals. Sesame oil was used by some as a solvent, an oil which is known to cause subcutaneous sarcomas occasionally. Other workers used a rather drastic chemical method for the preparation of the test material, which leaves some doubt as to whether the carcinogenic material was preformed. Final proof must wait, therefore, until the agents are isolated in chemically pure form and until it is shown that the alleged active compounds occur in the body in sufficient concentration and can act over a sufficient time to induce tumors. This line of investigation serves as an illustration of the great effort and time which are required to established fundamental facts in cancer research.

Brief mention should now be made of work designed to explain the difference in behavior of malignant and normal cells. Here again the biochemical approach seems to be advantageous since it is reasonable to suppose that the difference in behavior may be due to differences in biochemical organization. One of the chief difficulties is the availability of a suitable control tissue for a given tumor (*17*). We have recently used with some success the normal and regenerating liver of the rat for comparison with a rat hepatoma. The regenerating liver serves as a comparison of a proliferating normal tissue with the proliferating liver tumor. The results so far obtained indicate that the tumor is characterized by a high anaerobic and aerobic lactic acid production and a respiratory quotient below unity,[3] by a very low content in riboflavin (*10*) and coenzyme (*11*), by a great reduction in glycogen (*6*) with an apparently unchanged amylase activity, and, most striking, by a reduction in the catalase activity to one one-thousandth of that of the normal control tissues (*9*). The activity of some other enzymes seems to be unchanged. In short, it appears that some of the important enzymatic activities of the tumor show marked decreases, which may perhaps account for the specific tumor biology. It is evident that whatever differences have been found are of a quantitative and not of a qualitative nature.

Another line of inquiry which in the past has been ignored deals with the systemic influence of cancerous growth as evidenced by cancer cachexia. Here again biochemistry may be helpful.[4] I have just referred to the great diminution of catalase in the rat hepatoma. Work at the Institute has shown that the catalase activity of the morphologically normal liver of rats carrying a large subcutaneous hepatoma transplant is reduced to less than one-tenth of that of the liver catalase of a normal rat. This effect is easily reversible, because after extirpation of the tumor the liver catalase returns to normal within 48 hours (*8*). Since catalase functions in the chain of oxidative reactions in tissue metabolism, it is rather interesting to find a very great reduction in catalase activity in the hepatic

[3] *Work carried out in the Department of Biochemistry, Cornell Medical School.*

[4] *The first indication of a systemic biochemical action of the growth of two malignant tumors was reported some years ago* (21). *It was found that the nonprotein sulfhydryl compounds in certain tissues of the tumor-bearing animals showed a progressive decrease with increasing tumor growth.*

tumor and a lesser decrease in the apparently normal liver of the tumor-bearing animal. Unpublished data indicate that this decrease in liver catalase occurs also with other rapidly growing malignant tumors. Further studies may lead perhaps to information of some use for correcting the pathologic cancer metabolism.

Time does not permit me to discuss research on diagnosis and treatment, and I must refer to my Barnard lecture of a year ago (*18*).

In conclusion, I have tried to formulate some phases of cancer research and to report in a general way on the progress which is being made. Emphasis has been placed on the study of carcinogenesis, since hope of preventing cancer, not only the so-called occupational but also the spontaneous neoplasms, must be based on such studies. I have also stressed the importance of work aiming at a better understanding of the behavior of cancer cells in comparison with the normal cells of origin. Great difficulties are met in the study of these problems in human beings. The limitations of purely morphologic studies were mentioned and the value of a physiologic and biochemical approach was pointed out. The outlook is good for further progress from combined laboratory and clinical research. Under present world conditions, the main responsibility for such studies for years will rest on American investigators.

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Tumor Immunity

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INTRODUCTION

The literature on tumor immunity is voluminous. From 1916 to 1938 seven reviews, or monographs, appeared. They dealt with all or some phase of this field in more or less detail. These seven papers by Tyzzer (*1*), Woglom (*2*), Murphy (*3*), Woglom (*4*), Caspari (*5*), Lumsden (*6*), and Clemmesen (*7*), taken as a whole, covered the essential knowledge of tumor immunity up to the time each was published. Even as early as 1916, Tyzzer said, "it is quite impossible to present within reasonable space a comprehensive review of all investigations in tumor immunity." Woglom's second review in 1929 contains a bibliography of over 600 titles.

Many of the problems of tumor immunity considered by the early investigators are still unsolved. For example, little is known as yet regarding the mechanism, if any, possessed by mammalian species of resisting malignant neoplasms arising spontaneously in their own bodies. On the other hand, a tremendous amount of information has been accumulated in the past two or three decades regarding the process of cancerization and the means possessed by animals of resisting the growth of an implantation of live cancer cells from another animal of the same or different species.

An effort will be made in the present paper to bring together and set forth as briefly as possible the more important observations discussed in previous reviews, to indicate where possible which of these observations have received general acceptance, and finally to consider only the more significant studies in tumor immunity which have been made in recent years. A review of the literature on immunity to tumors associated with filterable agents is not included.

GENERAL CONSIDERATIONS CONCERNING TUMOR IMMUNITY

It seems now well-established that immunity to implanted tumors gives no assurance against the subsequent development of spontaneous tumors.

The fact that tumors may be transplanted into a normal animal as readily as into one in which a spontaneous tumor has developed and that young, healthy animals are more favorable to the growth of an implanted tumor than old or sick animals, suggests that there is no abnormality of the growth-regulating mechanism of the body as a whole, but rather a local derangement of the group of tissue cells from which the tumor arises.

Spontaneous regression of a spontaneous tumor is extremely rare, although it is claimed that a number of such recoveries among human beings are well authenticated. A series of these cases have been analyzed by Rhodenburg. However, the record is not clear as to whether or not these spontaneously recovered persons were immune the rest of their lives to the development of cancer at some other or the same site. It should be pointed out, too, that in the strict sense of the term there is no such thing as a spontaneous cancer or a spontaneous regression. Every event in biology, as in physics or chemistry, has an efficient cause. Indeed, the analysis of these recovered cancer cases showed that the majority of them followed some febrile condition or partial extirpation. As rare as these cases are, the fact that they occur at

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all is, we believe, highly significant, because their occurrence indicated there was a mechanism of some kind by which the complete regression of the tumors was brought about. Finding this mechanism is the job of the cancer investigator.

The pathologists can distinguish a great variety of reactions on the part of tissues surrounding a tumor: some are antagonistic, others indifferent, still others unquestionably favorable. Tyzzer reports that certain uterine carcinomas excite a pronounced infiltration of eosinophiles, and in such cases the reactionary tissues may exceed in amount the essential tissue of the tumor.

In the general dissemination of metastatic emboli of tumor cells throughout the animal body, certain organs may remain free. This has been called "organal immunity," and sometimes nodules of tumor cells have been found in such organs undergoing degeneration. The spleen, the heart, striated muscle, and the kidneys are the organs generally considered to be most resistant to metastases.

As a rule, tumors that metastasize in the individual in which they arise have a greater tendency to metastasize when successfully transplanted to other animals. There is, however, no evidence that the presence of spontaneous tumor may stimulate a reaction which is unfavorable to the development of tumor emboli.

It is now universally believed that immunity to a transplanted tumor is the reaction of an organism to a foreign cell and that the degree of immunity is proportional to the foreignness of the cells. Dead tissue or tissue products of mammalian tumors do not immunize—living cells seem to be necessary at the present time.

Tumors are readily re-implanted in the individuals in which they arise—*autoplastic grafting.*

Implants into individuals of the same species result in a variable number of failures, depending upon the genetic constitution of the test animals—*homoplastic grafting.*

Invariable failure follows implantation of tumor—*heteroplastic grafting*—in animals of another species, except under special conditions noted later.

When a tumor is implanted into an individual of an alien species or strain, the tumor may grow for the first 5 or 6 days as readily as in animals of the same species or strain, and the host provides stroma and blood vessels for the implant. At the end of this period, however, an inflammatory reaction appears, and a cellular exudate accumulates in amounts sufficient to interfere with the blood supply and to isolate the tumor. Regression occurs, and subsequent implants are disposed of more promptly.

Genetic studies have been made by Tyzzer, Little, Strong, and others, in which the various types of crosses between highly inbred strains of mice have established beyond any reasonable doubt that susceptibility and nonsusceptibility to transplanted tumors are dependent upon a complex of independently inherited unit factors. In general, whether or not a transplanted tumor grows depends upon a compatible relationship between the reaction of the host, as determined by its genetic factors, and that of the transplanted tumor cell, controlled by its genetic constitution. Thus, a tumor arising spontaneously in one member of a highly inbred strain will grow when transplanted into another member of the same inbred strain since the donor and recipient have the same genetic constitutions. Likewise, a tumor arising in either of two parent inbred strains usually will grow in their F_1 hybrid since the hybrid has a complete complement of the genes occurring in each parent strain (sex-linked genes excepted), but the trans-

plantation of a tumor arising in an animal of the F_1 generation into either parent strain is seldom successful since neither parent strain possesses all genes found in the F_1 hybrid.

In some cases changes have occurred in the genetic constitution of transplanted tumors. These have been defined as mutations; and since the tumor tissue is somatic tissue, they may be properly termed "somatic mutations." Certain investigators feel that the transformation from the normal to the cancer cell at the very beginning is a somatic mutation. If this is true, the antigenic power of the tumor cells might, therefore, be proportional to the degree of foreignness.

At this point it seems natural to ask, May not this deviation of the cancer cell from the normal in some cases be sufficient to yield a slight degree of antigenicity and thus explain the occasional spontaneous regressions? The studies of MacDowell, Potter, and Taylor (*8*) and Furth and Barnes (*9*), which are discussed later, also support the theory that cancer is a somatic mutation.

The conception of Tyzzer in 1916, regarding the nature of tumors and their relationship to the other tissues as varying degrees of foreignness, appears to be still valid:

(1) The interaction of the normal tissues are mutually beneficial so that their relationship is one of *symbiosis*.

(2) Benign growths and anomalies, while not distinctly harmful, are usually of no benefit to the individual. The relationship is one of *commensalism*.

(3) The malignant tumors are *parasitic* in nature, since they develop at the expense of the other tissues of the body. They are so adapted for growth that once established, they seldom arouse any effective resistance on the part of the body. There is some evidence, however, of a local reaction of tissues in some types of tumors which is unfavorable to tumor growth.

The observation that an animal in which a transplantable tumor has receded is often resistant to a second inoculation was at first considered to be a fact of great importance until it was later discovered that previous treatment with a normal tissue of the same species is effective in producing resistance to some transplantable tumors but not to all. Certainly up to the present, no one has been able to make an animal refractory to the inoculation of a graft from its own tumor. When attempts were made clinically to cure neoplasms in man with various preparations of viable autologous cancer cells, such cells often survived and gave rise to new tumors. Nor were attempts successful to make animals resistant to tumor inoculation by treatment with the spleen or other tissue from their own bodies.

The fact that primary cancer of the spleen is rare had led many investigators to use extracts of spleen or concentrations of this organ from normal and from immune animals. However, it has been pointed out by others that the kidney and heart both have less metastases than the spleen and that if the spleen has some anticarcinogenic substance, the kidney should have more. This type of therapy has certainly not yet reached the stage of clinical usefulness.

In his second critical review (1929), Woglom (*4*) pointed out that tumor immumity cannot be compared with the immunity produced against the invasion of bacteria. "It is the difference" he says, "between fighting a traitor within the body and repelling an invader from without." Hence he does not believe there is "the slightest reason to anticipate a cure by the production of antibodies." An imposing number of observations have been made which seem to discourage thoroughly any hope one may entertain of

developing a tumor therapy based on immunity principles. We list them as follows:

(1) After a transplanted tumor is once established in a foreign soil, its growth cannot be effected. The injection of normal homologous tissue is effective in preventing the growth of a new graft but entirely without effect upon the growth of an established tumor. "How, therefore", it is asked, "can one expect any method to be effective against a spontaneous tumor which is so much more favorably situated?"

(2) Transplantable or propagable tumors vary a great deal in their ability to induce immunity. Even the same tumor will vary from time to time in its ability to induce immunity depending upon the growth energy of the tumor and the soil in which the tumor is implanted.

(3) Extensive central necrosis is a wellnigh constant feature of all transplantable tumors. A receding tumor like any growing one is made up usually of a necrotic central portion and a marginal zone of healthy cells. These marginal cells are the last to die, as a rule. One would expect them to be the first to die if there is a general immune reaction on the part of the host that brings about the disappearance of the neoplasm. Not only do these marginal cells come in contact with the tissues of the host and therefore with any hypothetical agent resident therein but they are the only cells of the tumor with an adequate circulation so that they are most exposed to any harmful substance, if such there be, in the blood stream. On the other hand, receding Jensen rat sarcomas have been observed in which there was a zone of necrobiosis sweeping inward from the margin and the neoplasm was thus gradually converted into a fibrous nodule.

(4) It not rarely happens that a tumor which has begun to regress recovers itself and at the next weekly charting is found to be growing again. Not so frequently, yet often enough to be taken into account in any explanation of the resistant state, a neoplasm will begin to recede, will next pass through a phase of renewed growth energy, and then finally undergo complete regression. This is not easily explained on the basis of a generalized change in the organism of the host.

(5) It is common to encounter nodules of firm, translucent, and apparently healthy tissue alternating with degenerated and cystic portions.

(6) A regressing transplanted tumor not uncommonly contains as many mitotic figures as an actively growing one, and it is sometimes impossible to distinguish under the microscope between a growing and a regressing neoplasm.

(7) Regressing neoplasms are still transplantable although proliferation is not so energetic and the number of successful transplanted tumors obtained from a disappearing tumor is small.

(8) Continuous growth of one tumor and the concomitant regression of another in the same host have been observed and do not support the idea that spontaneous cure is due to some generalized change but rather that there is a local alteration in the tumor. In this connection, however, some later observations have shown that bilateral Jensen rat sarcomas of the same age pursue a similar course, either both grow or both recede.

Because of these observations, the emphasis was shifted for a time from a consideration of generalized immunity as the cause of tumor regression to the blood supply of the tumor. Interruption of the circulation or inefficient circulation, it was felt, may account for some cases of spontaneous cure. In other words, thrombosis may be primary and necrosis secondary to regression. Perhaps the growth or

regression of a tumor becomes, in part, a question of the extent to which its vessels have been obliterated by thrombosis, and the difference between a growing and a receding neoplasm is a difference only in degree and not in kind. Retrogressions of neoplasms are sometimes noted after a sharp attack of fever caused by pneumonia and other acute infections in which the viscosity of the blood and the amount of fibrinogen is increased. The vascular endothelium, too, may be injured by bacteria and their toxins, thus providing optimum conditions for thrombosis.

There are several other points about immunity to transplantable tumors which should not be overlooked by investigators who may attempt to work in this field. These may also be briefly set forth:

(1) The length of life of the host following the transplantation is not a measure of virulence of the tumor. An animal with a small ulcerated tumor may die sooner than one with a large, intact growth.

(2) Natural and racial resistance are hereditary.

(3) Acquired immunity reaches its maximum about the tenth day, is uniformly distributed throughout the body, and persists for 2 or 3 months.

(4) Acquired immunity is neither hereditary nor passively transferred. (See observations of MacDowell and Potter, p. 329, for recent report of exception.)

(5) An acquired immunity is valid against some other propagable tumors but not all.

(6) No antibodies in the blood stream of either naturally immune or artificially immunized animals comparable with those which the body elaborates against bacterial invasion have been discovered.[1]

(7) The agent or agents that cause regression of tumors when implanted into a foreign species are not in the blood stream, for mouse and rat tumors can be grown *in vitro* in the plasma of all alien species except in that of the goat.

(8) While the variations in susceptibility to implanted tumors among different races of the same species is due unquestionably, in part, to heritable constitutional causes, nevertheless the growth energy of the tumor itself plays an important role in determining the outcome of implantation. For example, strain resistance to the highly malignant Crocker Institute mouse sarcoma 180 is usually absent. Nearly all mouse strains are susceptible to this tumor and vary only in degree.

(9) Tumor resistance can be induced only with homologous tissues. Most attempts to immunize rats with chicken sarcoma or chickens with rat sarcoma have failed. Yet occasional reports have been made of producing immunity with heterologous tissues. All homologous organs and tissues will immunize, and embryo skin is considered to be the most efficient. The potential vitality of the tissue in the new host may be related to its immunizing ability, but generalizations are not possible at the present time.

(10) It is practically impossible to separate natural from acquired immunity, because without the test of implantation there is no way of telling whether or not an animal is resistant. Natural immunity is believed to be the power to react so promptly with a defensive mechanism that the graft is unable to obtain a foothold. The difference between the two types is perhaps quantitative only.

Transplantable new growths may be divided, according to Woglom, into five types on the basis of their growth behavior in the new host: (1) The progressive tumor which steadily increases in size; (2) the retarded tumor, the growth rate of which

[1] *The work of Lumsden* (6), *who claimed to have demonstrated specific anticancer antibodies, is considered later, p. 324.*

diminishes gradually as the tumor increases; (3) the oscillating tumor, the growth of which oscillates within narrow limits for long periods; (4) the steadily disappearing tumor; and (5) the tumor which recedes until about to disappear and then increases steadily in size again.

Some observers think that the factor responsible for regression resides, at least in part, in the tumor cell itself. Woglom concludes his 1929 review with the apparently justifiable generalization that—

> the outcome of tumor inoculation is determined by an interplay between the hostility of the host and the proliferative vigor of the implant, hence an absolute immunity does not exist. Resistance is effective during the first few days following inoculation but entirely powerless against an established tumor. * * * nothing may accordingly be hoped for at present in respect to a successful therapy from that direction.

Little, if anything, has been added to the literature in recent years which would tend to change this pessimistic point of view.

THE LYMPHOCYTE IN IMMUNITY

That the lymphocyte plays a role in tumor immunity was suggested by the fact that a large number of these cells gather around any tissue capable of eliciting immunity, while dead tissue (*4*) excites no lymphocytic response. Furthermore, there is no lymphocytic response when an immune animal is injected for a second time with tissue capable of inducing the refractory state. The lymphocytic reaction is apparently a defensive mechanism against both tumor grafts and those of normal tissue and is perhaps an index of the foreignness of the graft. It is more pronounced in homotransplants than in autotransplants. While DaFano first called attention to the lymphocyte as significant in the animal body's response to tumors, the lymphocytic hypothesis is most intimately associated with the name of Murphy. In his monograph Murphy (*3*) reviews the original reports of his investigations which appeared over a period of 14 years (1912–26) and which cover a more or less continuous account of the development of knowledge regarding the role of the lymphoid type of cell in the mechanism of tumor regression. Murphy observed that the avian embryo had no protective mechanism against the growth of heteroplastic tissue until after the eighteenth day of incubation when a method of defense was rapidly developed which attained full force at the time of hatching. In an attempt to discover what tissue or organ was responsible for the resistance, mammalian-tumor grafts were transplanted into the embryo together with bits of normal adult chicken tissue. The effects of spleen were striking. The tumor was found to be largely necrotic and encircled with small round cells. Marrow had a similar action though less complete, and either would inhibit growth of the graft even though placed at some distance from it after it had become established. All other tissues examined proved to be inert.

Murphy also found that chicken and mouse tumor would grow in rats after the lymphatic system had been largely depleted by large doses of X-rays. The lymphocyte proved to be equally essential in immunity to homologous tumors; resistance disappeared when the lymphocyte was eliminated by appropriate X-ray dosage. Repetition of these tests on animals with spontaneous tumors confirmed the results with transplantable neoplasms. Murphy believed that the round-cell infiltration, so characteristic of the reaction to foreign-tissue grafts, is a purposeful reaction and that these cells are the agent through which the mechanism exerts its force. He found that the avian embryo and the adult brain are without defense against heteroplastic grafts and that there

is a notable absence of the round-cell reaction in both cases. These tissues are, however, rendered completely immune when supplied with lymphoid tissue.

Other observers do not attribute so much importance to the lymphocyte as does Murphy. Their view is based on the following observations:

(1) Decreasing the circulating lymphocytes by small doses of X-ray does not render mice more susceptible to the inoculation of a spontaneous tumor from another mouse of the same strain, nor are mice with spontaneous lymphocytosis resistant to the implantation of a primary tumor from another mouse of the same strain.

(2) The normal peritoneum of the white mouse is rich in cellular elements, 115,000 to the cubic millimeter, of which about 55 percent are lymphocytes. Mouse tumor implanted into the peritoneum of these mice grows rapidly.

(3) A rat with an extraordinarily high lymphocyte count might have, nevertheless, a growing Jensen sarcoma, whereas another with a count but little above normal might be immune.

(4) Small round-cell infiltration has been seen in both growing and receding spontaneous carcinomas of the mouse.

(5) The death of heterotransplants is not always accompanied by the presence of lymphocytes. Sometimes they are absent.

(6) Transplants to the spleen have resulted in successful takes. Some investigators claim that the transplants take better than in the liver. Tumors placed in the peritoneal cavity have invaded the spleen readily. When spleen is mixed with tumor before inoculation, tumor growth has been embarrassed but other tissues also inhibit. The spleen from an immune rat has not affected unfavorably the *in vitro* proliferation of tumor cells. Splenectomy neither accelerates nor retards the growth of propagable rat tumors. No relation has been observed between splenic enlargement and the resistance to tumor implantation.

In this connection, the experiments of Lewisohn (*10*) should be recorded. He prepared a concentrated beef-spleen extract in which 1 cc. represented 100 gm. of spleen. The first doses of a daily series of subcutaneous injections of this concentrate were made in Rockland Farm strain mice 7 to 9 days after the transplantation of sarcoma 180. After 3 or 4 injections of the extract, marked hemorrhages occured in the tumors of the majority of the treated mice. Of 281 animals treated, the tumor disappeared completely in 170 (60 percent). Corresponding controls showed a spontaneous regression in 8 percent of 290 animals. Extracts from the liver, heart, pancreas, and testes failed to cause hemorrhages in the tumor or to influence its growth. As far as is known, confirmation of these results has not been published.

NECROHORMONES

Despite the fact that only living-tissue implants will immunize, Caspari (*5*) and his associates believed that the death of the tumor cells was the essential feature in the process. The necrotic material, it was claimed, exerted four functions: (1) It excited the formation of stroma and capsule; (2) it nourished the tumor cells; (3) it exerted a toxic effect; and (4) it called forth a general immunity. He believed resistance was due, not to living cells, but to chemical substances (necrohormones) elaborated from dying cells. His hypothesis has not been generally accepted, and the majority of investigators do not believe that the known facts warrant this hypothetical conception of "necrohormones."

SERUM ANTIBODIES

Although the majority of investigators have been unable to demonstrate in the blood the existence of specific anticancer-cell antibodies similar to antibacterial antibodies, Lumsden (*6*) believed, as a result of his investigations, that "there is no antibody whose existence is more definitely proved than that of these anti-malignant-cell antibodies." He found that antisera could be produced in any animal by treatment with heterologous tumor. Such sera contain antibodies which are toxic to malignant cells from any species. The toxic effect is readily demonstrated upon cancer cells growing *in vitro* but is comparatively ineffectual *in vivo* because the serum does not reach the cancer cell in sufficient concentration. Lumsden inoculated the Jensen rat sarcoma into the feet of rats; and just before antiserum was injected into the resulting tumors, a rubber band was applied to the ankle and allowed to remain for 2 or 3 hours so as to occlude the blood vessels. In every instance the treated neoplasms regressed, and the animals subsequently were resistant to the Jensen sarcoma. In criticism of this work it has been pointed out by Woglom and others that this tumor is notoriously predisposed to spontaneous regression. Thus the tumor immunizes against itself without the aid of an antiserum. However, Lumsden claimed to have had only 5 regressions out of 50 animals in the controls. He found also that an implanted tumor already established in the body can be gradually destroyed by injecting antiserum or formalin into it, and thus active immunity against a tumor is induced by a mechanism which may be called "auto-vaccination."

Lumsden has postulated five types of antibodies in relation to tumor immunity:

(1) Anti-species antibodies: When rat cells are injected into a rabbit, antibodies toxic to rat nucleated cells are evoked.

(2) Homologous cytotoxins (homocytotoxins): These cytotoxins are produced when the cells of one rat are inoculated into another rat. They are also found frequently in the sera of rats with regressing tumors but not in rats with progressively growing tumors.

(3) Normal heterocytotoxins: Naturally existing cytotoxins are present in the serum of normal untreated animals and are lethal to the cells (*in vitro*) of any heterologous animal.

(4) Autocytotoxins also exist, but the only known example of these are autospermatoxins. The spermatozoa of a rat are agglutinated and killed by the individual rat's own serum.

(5) Toxic agents are present in the serum of many tumor-bearing animals and very rarely present in the serum of untreated normal control animals. Unlike the other cytotoxins which kill in a few minutes, this cytotoxin causes no apparent damage in tissue cultures of malignant cells for 2 or 3 hours, but thereafter a varying and often a large percentage of the cells become spherical and die gradually.

The demonstration of specific anticancer-cell substances in the blood of cancer-immune animals is not generally accepted, for it has been shown that even when susceptible and naturally immune rats are united in parabiosis no transfer of the protective agent has occurred. Nor have antibodies been demonstrated by means of the anaphylactic technique which is perhaps the most sensitive and specific method of all in detecting such substances in the sera of immune animals. Furthermore, Phelps (*11*) has shown that the antibodies of the so-called anticancer sera of Lumsden have no specific action on cancer cells and are not peculiar to malignant cells but are probably iso-antibodies formed in response

to injecting of foreign though homologous cells. There is no indication that such an antiserum could be therapeutically useful.

Lately, Mann and Welker (*12*) claimed that they had demonstrated specific precipitin antiserum prepared from proteins of carcinomatous tissue. These specific carcinoma proteins may appear in the blood stream of the patient with carcinoma and be detected with the appropriate antiserum prepared by injecting rabbits with ground carcinomatous tissue adsorbed by aluminum cream. The antiserum did not react with normal human tissues.

Hoyle (*13*) reported that alcoholic extracts of certain mouse tumors, namely, S37, tar carcinoma 2146, and the Mal sarcoma gave complement fixation with the sera of mice bearing these tumors. The reaction may be similar to that described by Herszfeld and Halber (*14*) in human cancer. The antigen involved appears to be lipoidal, and those produced by all three tumors are closely allied and probably identical. No antigen is present in normal mouse tissue and no antibody occurs in the sera of normal mice.

SUCCESSFUL HETEROTRANSPLANTATIONS

Clemmesen's experiments (*7*) seem to prove that resistance even to heterotransplants can be temporarily broken down. He caused rats to be susceptible to the inoculation of mouse tumors by *in toto* exposure to strong X-rays. That actual growth occurred was proved by the size of the tumors and by the fact that the musculature of the rat became destructively infiltrated. This effect was not strong enough to create conditions for a lasting growth of the mouse tumors in the X-rayed animals. After 2 to 4 weeks the grafts became necrosed. The X-ray seemed to delay the mobilization of the animal's defensive powers and the process of self-immunization. On the other hand, X-rays are known to increase the incidence of spontaneous tumors. Yet it must be remembered that there is no similarity whatever between the manner in which a spontaneous tumor arises and the development of a transplantable tumor as the result of grafting.

Putnoky (*15*) was able to propagate the Ehrlich transplantable mouse carcinoma continuously for 7 years in rats. It is said to be one of the most rapidly growing of transplantable mouse tumors. It could not be transplanted to rats previously treated with mouse tissue whether normal or cancerous, while a previous treatment of rats with rat tissue did not produce such resistance. The tumor in the rat behaved as if it were still composed of mouse tissue and could always be readily transferred back to mice. In mice it never regressed and grew so vigorously that no resistance could be induced in mice to its transplantation, whereas in rats growth ceased after 10 days and regression occurred. Over many years the propagation of this rat strain of mouse cells in a rat has not transferred the property of malignancy to the cells of the rat which served as host. Resistance was induced against heterotransplantation by previous inoculation of tissues homologous to the tumor, in this case mouse tumor, but not of tissues homologous to the host, in this case rat tissue. There was some evidence, however, that the mouse strain and the rat strain of the Ehrlich mouse carcinoma were biologically different. Rats in which the rat strain had grown were resistant to subsequent transplantation of tumors of rat origin, the Jensen sarcoma and the Walker rat carcinoma, while rats in which the mouse strain had grown were less resistant.

A new strain of the Ehrlich mouse carcinoma was successfully transplanted into

young rats by De Balogh (*16*) in October 1938. This new heterologous tumor was designated "Budapest 1938." Preliminary antiphylactic experiments suggested that the mouse tumor in its thirtieth to fiftieth passage in the rat was not unchanged but possessed properties also of rat protein. This is not surprising since the tumor stroma of an implanted tumor is derived from the host.

Although tumors are propagable as a rule only in the species in which they originate, successful transplants have been made to the embryos of very closely allied species by a number of investigators.

Successful transplantation of human cancer into the anterior chamber of the rabbit eye has been reported by Greene and Saxton (*17*), but they have not developed their technique to the point where it has become a useful and dependable experimental tool.

IMMUNITY INDUCED BY CUTANEOUS INOCULATIONS

Both experimental studies on animals and statistical studies on human beings have been made to detect some relationship between the presence of skin tumors and generalized immunity.

Andervont (*18*) demonstrated that a single caudal inoculation of the mouse sarcoma 180 induced concomitant immunity in about 60 percent of adult mice. This resistance reached its peak in about 2 weeks after tail inoculation. Highly susceptible mice were unable to acquire resistance to the same extent as animals which possessed some degree of natural immunity. Immunity induced by the tumor was also effective against mouse adenocarcinoma No. 63. Hence, the immunity is not specific to sarcoma No. 180. In a later study, Andervont (*19*) using eight strains of highly inbred mice showed that there was no correlation between the susceptibility to spontaneous mammary tumors and susceptibility to the induction of subcutaneous growths.

Andervont (*18*) found that immunity to sarcoma 180 was more readily induced by caudal inoculation in female than in male mice.

Besredka and Gross (*20, 21*) reported immunity in some cases following intracutaneous inoculation of tumors.

Wilner and Zakrzewski (*22*) attempted to duplicate the work of Besredka and Gross with mouse sarcoma 37 but found no immunity or regression of any inoculated tumor.

Gross (*23*) found that neoplasms induced in mice, rabbits, and chickens by intracutaneous inoculation of emulsions of the Ehrlich sarcoma, Brown-Pearce rabbit carcinoma, and the Rous sarcoma, respectively, sometimes regressed spontaneously, and the animals thereafter displayed a specific generalized immunity to the inoculation with those tumors. But the immunity was not transferable by blood serum or organ emulsions to other animals. Regression following subcutaneous inoculation of similar doses was unusual.

Gross (*24*) has reported that female mice are more resistant than males to an intradermal implantation of a small dose of a mouse sarcoma as evidenced by the smaller incidence of takes and greater incidence of regression in the females.

Saphir and Appel (*25*), employing the Brown-Pearce carcimona, demonstrated that regressing cutaneous tumors cause a regression of coexisting and pre-existing testicular tumors and their metastases. The regression of the tumors and their metastases occurred only in those animals in which the skin tumors had begun to disappear, whereas in those instances in which the skin tumors showed no evidence whatsoever of regression, the spread of the tumor was not interrupted and the disease

progressed until it produced a fulminating carcinomatosis from which the animal died. Regression of the cutaneous tumors could be taken as an indication that the testicular tumors and their metastases would within a short time begin to undergo similar regression.

In appraising this work, it should be remembered that there are no pure inbred strains of rabbits and hence regressions in this case may have been due to natural resistance of the individual rabbits rather than to the effect of the intracutaneous tumors. The Brown-Pearce carcinoma displays frequently a high spontaneous regression rate, according to some who have had a long experience with it.

Fischer-Wasels (*26*) was able through intracutaneous injection of a virulent tumoral material to immunize rabbits against the Brown-Pearce carcinoma.

In a discussion of the incidence of cancer at different sites, Peller (*27*) concluded that the death rates in certain occupational groups from cancer of various organs conflict with the theory of the local origin of the disease. From an analysis of the mortality statistics of England and Wales, he concluded that an increase of carcinogenic irritation leads to an increase of cancer at the irritated spot but that not only is there no corresponding rise in the total incidence but also that this increased irritation in fact leads to a decrease in the incidence of cancer in some of the other organs. He therefore raised the question as to whether the production of a primary tumor in one organ does not bring immunity to others.

In a subsequent study with Stephenson (*28*), Peller examined the incidence of cancer in the United States Navy and reached similar conclusions. It was found that men exposed to the sun's rays, open air, and salt water have a skin- and lip-cancer rate considerably above normal and a greatly diminished incidence of all other cancers.

However, Conrad and Hill (*29*) made a comparative study of the incidence of mortality from cancer of the skin and lip and mortality from cancer of other sites in different occupational groups using in part Peller's own data. They were unable to confirm the reported inverse relation between cancer of the skin and lip and cancer of other sites but found, on the contrary, that there is on the average a slight direct association. Where a relatively high skin and lip rate was found among persons engaged in certain occupations, there was also à tendency toward excess of cancer of other sites.

Warren and Gates (*30*), on the basis of 1,149 carefully studied cases of cancer of the skin found in this same group more cancer of organs exclusive of the skin than would be encountered in a similar population drawn from Massachusetts at large.

RECENT MISCELLANEOUS OBSERVATIONS

Substances in normal tissues inhibitory to the growth of cancer have been observed. Schabad (*31*) reported that the addition of splenic tissue from normal mice inhibited the growth of the Ehrlich mouse carcinoma; whereas spleen from mice with transplanted induced or spontaneous carcinoma or sarcoma lacked this effect. The same difference was observed in the case of blood. When the serum of 60 cancer patients and 30 persons free from cancer was examined it was found that here again the serum of the former group had in most cases lost its inhibitory action.

MacFadyen, Sturm, and Murphy (*32*) found an agent in mouse and rabbit placenta which would inhibit the growth of transplanted mouse carcinoma. The mammary glands from pregnant rabbits or cows proved to have a similar factor.

Mammary tissue of pregnant rabbits was found to contain an agent which inhibited the growth of Bashford adenocarcinoma No. 63 in mice, but this material was without effect on Crocker sarcoma No. 180. Of the tissues so far studied, the mammary gland of rabbits had yielded the most active product. The factor was found only in the protein fraction obtained from the aqueous extract by full saturation with ammonium sulfate.

Casey (*33, 34*) has observed aggressin-like substances specific for the Brown-Pearce rabbit tumor. Slowly frozen and anaerobically preserved metastases or primary growths of the Brown-Pearce tumor were emulsified in normal saline. A single injection of 0.3 cc. of the emulsion was made intracutaneously or intratesticularly into rabbits 2 weeks before tumor transplantation. Such treatment resulted in a greater incidence and volume of primary tumors, a greater incidence, number, and volume of metastases, an increased mortality, and a decreased longevity. The material responsible for the phenomenon (sometimes called the XYZ phenomenon) was homologous, filterable, desiccable, thermolabile, and capable of preservation for several months. It differed from the Duran-Reynals factor (*35, 36*), which is a testicular extract having the power of increasing the invasion of the animal body by toxins, bacteria, and viruses. The latter factor does not affect the incidence, volume, or distribution of primary or metastatic Brown-Pearce tumors. The X Y Z factor does not affect the growth or spread of Bashford carcinoma No. 63 or tumor No. 180 of the mouse.

Lewis (*37*) failed to bring about immunity to induced tumors in pure inbred strains of mice by the absorption of tumor proteins (grafts of viable regression tumors, supernatant fluids from centrifugalized suspensions of viable ground and crushed tumor cells and tumor tissue inactivated by means of heat, alcohol, distilled water, and chemicals) and of other proteins from immune mice. Furthermore, the growth of a graft of any one of the induced tumors for 4 or many days in mice of the same strain as that in which the tumor originated, failed to bring about in the host immunity from the growth of a graft of the same or of another tumor. Since Lewis used inbred strains, the homotransplantations in these tests were different in only a slight degree from autotransplantations.

Resistance to transplantable lymphatic leukemia by the injection of embryonic tissue prior to inoculation has been obtained in rats by Sturm (*38*).

Barrett (*39*), employing two pure-line tumors, found that no significant resistance could be induced in an inbred mouse against a tumor derived from the same strain. When resistance to transplantable tumors was induced in susceptible mice by subcutaneous injection of whole blood, the degree of resistance varied with the genetic interrelationship of the host, the tumor, and the donor (of the blood).

Furth and Barnes (*9*) have shown that leukemic cells from spontaneous and induced leukemia in inbred mice may differ genetically as shown by their ability to be engrafted or failure to be engrafted on parental leukemic and nonleukemic stock. This genetic difference can be explained only by assuming that the cells of the induced leukemias are mutants.

MacDowell, Potter, and others (*40*) induced active resistance to experimental leukemia in mice by means of sublethal doses of living leukemic cells, and this resistance could be passively transferred to susceptible mice by the use of saline suspensions of minced tissue from actively immunized animals. Serum, however, was ineffectual in transferring passive immunity. Genetically identical living cells from

nonimmunized mice gave no protection. The authors state:

> This is probably the first time that any type of induced resistance has completely suppressed a neoplastic growth transplanted before the protecting treatment was given.

Later, MacDowell, Potter, and Taylor (*8*) found that an inbred strain of mice (C58) with a spontaneous leukemia rate of nearly 90 percent could be immunized effectively against the highly virulent cells of a long-established line of transplanted leukemia that started from a spontaneous tumor in a mouse of the same inbred strain. However, the high rate of spontaneous leukemia in C58 mice was not significantly affected by the immunization against the transplantable leukemia. These results were interpreted to mean that the induced resistance to highly virulent long-transplanted leukemia cells depends upon properties developed in the course of transfer, in other words, the development of a mutation. This observation, along with that of Furth and Barnes cited above, seems to be important immunological evidence in support of the mutation theory of cancer.

Perhaps here it should be noted that the leukemias are classified usually as cancers of the blood-forming organs for the following reasons:

(1) They are not transplantable from one strain of mice to another except in inbred strains.

(2) Intact cells are necessary to effect a successful transplant.

(3) They may be induced in mice with the same carcinogenic chemicals that induce true neoplasms.

(4) The microscopic pathology of leukemic cells resembles that of cancer cells.

(5) Resistance to transplantable leukemia has been induced by the injection of normal homologous tissues (*41*).

Eisen and Woglom (*42*) showed that rats of a highly inbred line which were uniformly susceptible to inoculation with a mammary carcinoma that arose spontaneously in a female of this line, could not be immunized against the tumor with embryo skin from the same line, though this material was highly effective in a partially related line. Thus, acquired resistance to transplantable tumors would seem to depend upon genetic differences between the animal inoculated and the one that originally produced the neoplasm and is not specifically directed against the malignant cell.

Barrett (*43*), working with the filterable chicken tumor I and using the method of differential sedimentation, observed that when products of the highest degree of purity obtainable were used there was a demonstrable immunologic difference between the extracts of chicken tumor and similar material from normal tissues. This observation is certainly suggestive that the filterable tumors are different from mammalian tumors in that their cells contain antigenic substances not present in normal chicken tissues.

Henle and Chambers (*44*) by means of high-speed centrifugation (25,000 r. p. m.) and differential sedimentation and the selection of the heavy particles, obtained antigens from the brain, liver, kidney, and testicle which were specifically agglutinated by the homologous antisera. Other organs gave indefinite or negative results. This method of detecting organ specific substances is applicable, it would seem, to the detection of cancer-cell-specific substances, if there be any. However, even if this work is confirmed—and there is no reason to doubt it— it may be quite a different thing from finding specific antigenic differences between, say, normal liver cells and cancer cells arising in the same organ.

DISCUSSION

Although qualitative chemical differences between normal and neoplastic tissue have not been discovered, one feels that such differences may exist since the behavior of cancer cells is different from that of normal cells. Is it possible for differences in behavior to rest on any other basis than a chemical one? If there is a chemical difference between normal and cancer cells, are our present immunologic methods—admittedly more sensitive in detecting traces of organic substances than the most refined chemical methods—capable of revealing such differences? Cancer tissue and homologous normal tissue undoubtedly have a large number of antigenic substances in common. These may completely mask small quantities of any specific substances in complement-fixation, precipitin, or anaphylactic reactions designed to reveal them. Therefore, careful fractionations, which will yield various components of normal and cancerous tissue in a high degree of purity, and the testing of these components for antigenic specificity may result in finding cancer-cell-specific substances. Such studies require the combined efforts of the biochemist and the immunologist.

On the other hand, since most investigators find only quantitative rather than qualitative differences between normal and neoplastic tissue, it is conceivable that immunologic methods are not applicable. There is no reason to believe that the behavior of the cancer cell as a whole can be explained satisfactorily on the basis of antigenic differences between its cellular components and those of normal homologous cell components, not all of which by any means are necessarily antigenic. The behavior of a cell may rest in part or perhaps *in toto* upon the pattern or configuration or arrangement of all its parts rather than upon specific chemical differences determinable by immunologic techniques. In chemical aggregates, it is well established that the sum of the parts is not always equal to the whole. If this concept is accurate, then we agree with Woglom that the quest for a method of cancer therapy by means of our present immunologic methods does not seem to be very promising at the present time. However, since immunologic methods are no less successful than other methods, there is no good reason to give up this line of approach.

In conclusion, it may be well to point out that significant *quantitative* differences have been reported recently in the chemical constituents of tumors and the normal cells of origin. Kahler and Davis (*45*) and Robertson and Kahler (*46*) have shown that the concentration of riboflavin, the prosthetic functional group of numerous enzyme systems, is much higher in liver than in hepatoma. Euler et al. (*47*) and Bernheim and Felsovanyi (*48*) have found low concentrations of coenzyme in tumor material, and several enzyme systems in liver tumors have been found to be quantitatively different from those in normal liver (Greenstein, Jenrette, Mider, and White (*49*)).

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Production of Tumors in Mice Following the Removal of Methylcholanthrene-Cholesterol Pellets

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Following the development of the cholesterol-pellet technique (*1*) for the induction of tumors (*2–5*) by carcinogenic compounds, it was considered desirable to ascertain if these pellets could induce tumors when kept in contact with tissues for short periods of time and, if so, what would be the shortest period necessary for induction of tumors in most of the animals.

Cholesterol pellets containing 5 percent of 20-methylcholanthrene (m. p. 179.5°–180.0°, corr.) and weighing between 12 and 15 mg. were inserted into the subcutaneous tissue of mice by means of a 12-gauge trocar. The pellets were placed subcutaneously in the lateral abdominal wall so as to facilitate their subsequent removal.

Removal was accomplished through a short incision in the shaved skin over one end of the pellet. If a pellet was encapsulated, the capsule was split and the pellet removed free from any macroscopically evident adherent tissue. Animals in which the pellet was soft or broken when removed were discarded. By this procedure those mice were eliminated in which a small piece of pellet might well have been left after the operation. No further treatment was given, and the incision usually healed within a few days.

Male mice of strain C3H, 3 months of age, were used. The high degree of susceptibility of the subcutaneous tissues of such mice to the carcinogenic activity of tumor-producing hydrocarbons had been established previously (*6*). They were fed an unlimited diet of Purina dog-chow pellets and tap water and were kept under similar environmental conditions.

Two experiments were performed: The first was undertaken in 1937 and was of a preliminary nature. The pellets were removed from mice in successive groups every 2 weeks up to the twelfth week after implantation. Fifteen mice which were allowed to retain their pellets served as controls. Tumors arose at the site of the pellet in all control mice. In all animals which were allowed to retain the pellet for 8 weeks or longer, tumors also appeared at the site from which the pellets had been removed. Four of five mice whose pellets were removed 6 weeks after implantation also developed tumors. In the second experiment, which was performed in 1940, 60 mice were employed. Pellets were removed from 20 mice 2, 4, and 6 weeks after implantation. Since the procedures in the two experiments were identical, the combined results are summarized in table 1. Both experiments were terminated 40 weeks after the implantation of the pellets.

The table shows that exposure to the pellets for 2 weeks induced tumors in 23 percent of the animals; exposure for 4 weeks gave tumors in 56 percent, and for 6 weeks in 88 percent of the mice. Of the 25 animals in the 6-weeks-exposure series, 2 died before the end of the experiment; only 1 survived for 40 weeks without developing a tumor. In an experiment now in

TABLE 1.—*The production of subcutaneous tumors in strain C3H male mice following exposure from 2 to 12 weeks to cholesterol pellets containing 5 percent methylcholanthrene*

[Numerators = number of mice developing tumors
Denominators = number of mice dying or killed without subcutaneously induced tumors]

Time pellets remained in subcutaneous tissues (weeks)	Number of mice	Occurrence of tumors, or death, after implantation of pellets (time in weeks)															Tumor incidence	Average time of appearance of tumors
		10	12	14	16	18	20	22	24	26	28	30	32	34	40	Total		
																	Pct.	*Weeks*
2	26	....	....	1/0	1/0	....	1/0	1/0	....	....	1/0	....	....	1/0	0/20	6/20	23	22
4	25	....	1/0	3/0	1/0	....	1/0	1/0	2/0	2/0	2/0	0/1	1/0	....	0/10	14/11	56	21
6	25	4/0	....	....	4/0	2/0	2/0	2/0	1/0	2/0	3/1	1/0	....	1/1	0/1	22/3	88	20
8 to 12	15	....	2/0	4/0	3/0	3/0	2/0	....	....	....	....	1/0	....	....	....	15/0	100	16
Controls (pellets not removed)	15	1/0	7/0	1/0	4/0	1/0	....	....	1/0	....	....	....	....	....	....	15/0	100	14

progress 16 mice were allowed to retain their pellets for 6 weeks; 13 (or 81 percent) developed tumors at an average time of 16 weeks after implantation. These findings show that 6-weeks exposure is sufficient for tumor induction in most C3H male mice.

Since no reliable methods were available for the determination of minute quantities of methylcholanthrene in tissue, no analyses were carried out on the tissues at the site of removal of the pellets for the presence of residual hydrocarbon, particularly since it has been found (7) that as little as 0.02 mg. of methylcholanthrene can produce subcutaneous tumors in 50 percent of strain A mice in 40 weeks.

All pellets removed in the first experiment were reimplanted into other C3H male mice. Tumors were induced in the new hosts by pellets in each group. The numbers involved were too small, however, to provide information as to whether or not those retained for only 2 weeks were more potent than those retained for 12 weeks.

This paper records a technique for tumor induction which may be useful in various kinds of investigations. It should be applicable in therapeutic studies in which induced tumors are desired and in which removal of the bulk of the tumor-inciting agent is required. The technique may also be useful in studies of the effect of diet upon the induction of tumors inasmuch as the influence of a dietary regime may be obscured by the persistent action of excess carcinogen.

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Mitotic Frequency in Methylcholanthrene Epidermal Carcinogenesis in Mice[1]

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The Barnard Hospital is attempting to determine the alterations that occur in as many properties as can be measured quantitatively in standardized methylcholanthrene carcinogenesis and to integrate the results with a view to building a comprehensive picture of modifications that lead to cancer in epidermal cells of mice (Cowdry and Paletta (*1*, *2*)). Mitotic frequency is considered in this paper.

Most of the previous studies of the effect of carcinogenic chemicals on cell growth were made on plant tissues, on protozoa, on the lower invertebrates, and on tissue cultures. Cook, Hart, and Joly (*3*) showed that the effect of 1,2,5,6-dibenzanthracene on the proliferation and respiration of yeast can be stimulative or inhibitory depending upon concentration. A concentration of 9×10^{-4} M caused a 50-percent increase in cell numbers, quantities both larger and smaller had less effect, and four times this amount was sufficiently toxic to be inhibitory. Dodge, Dodge, and Johnson (*4*), using the dry weight of yeast cultures as an index of growth, found only small differences between cultures to which methylcholanthrene had been added and the controls. The methylcholanthrene-treated cultures tended to have a slightly longer lag phase and slightly less dry weight. Owen, Weiss, and Prince (*5*) found that benzpyrene, dibenzanthracene, and methylcholanthrene in their water-soluble form stimulate slightly the growth rates of tissue cultures of pea rootlets.

Mottram (*6*), using 3,4-benzpyrene in a 1-to-1,000,000 dilution, and Spencer and Melroy (*7*), using various dilutions of a stock solution of 0.1 percent methylcholanthrene in benzene, found that these carcinogens produced an increase in the rate of cell division of paramecia when they were added to the culture media.

Studies on the lower invertebrates have tended to show that the carcinogenic hydrocarbons exert a stimulating effect upon cell growth. Hammett and Reimann (*8*, *9*) found that both 1,2,5,6-dibenzanthracene and methylcholanthrene stimulate proliferative activity in the marine hydroid, *Obelia geniculata*. In the aquatic worm, *Euplanaria dorotocephala*, which has the ability to regenerate an entire new animal from a cut part, Owen, Weiss, and Prince (*5*, *10*) showed that dibenzanthracene, benzpyrene, and methylcholanthrene all increase the rate of reconstitution of head and tail segments and the reproduction of whole animals.

On the contrary, studies on the effect of the carcinogenic hydrocarbons on the rate of growth of cells in tissue cultures have for the most part indicated an initial inhibitory effect of methylcholanthrene on cell division. Magat, Lebenson, and Volkeson (*11*) found that a lard solution of 1,2,5,6-dibenzanthracene exerted no stimulating effect on the growth or on the carbohydrate metabolism of mouse and fowl tissues *in vitro* in from 2 to 18 days. This period was considered too short for the production of malignant change but sufficiently long to insure penetration of the hydrocarbon into the cells. Earle and Voegtlin (*12*) and Voegtlin (*13*) studied

[1] *Aided by a grant from the National Cancer Institute and an anonymous donor.*

the effect of dibenzanthracene and methylcholanthrene on rat and mouse fibroblasts in tissue cultures and found that they cause severe growth retardation and degeneration of the cultures, the effect being roughly proportional to the concentration of the reagent and the time of exposure. They observed no stimulative action of either of the carcinogenic hydrocarbons used in their experiments.

The variety of responses obtained by different investigators may be due to a number of factors such as the species of animal used, the varied physiological response of different types of tissue, the concentration of the carcinogen employed, and the method of its application.

Apparently few, if any, studies have been made on the effect of methylcholanthrene on cell division in mammals. As far as we are able to determine, none of the previous reports are based on quantitative measurements. The present experiment was undertaken to determine by actual counts of mitotic figures the effect of methylcholanthrene on cell division in the epidermis of the mouse.

MATERIALS AND METHODS

The epidermis of the ear of the mouse was chosen as a suitable tissue for the present study since it lends itself readily to the preparation of whole mounts by the method mentioned by Hoepke (*14*) and given in detail by Cowdry (*15*). The epidermis can be separated completely from the dermis after placing the tissue in a 1-percent aqueous solution of acetic acid in the ice box for 24 hours. The hair follicles in the ear of the mouse are smaller and fewer in number than on the rest of the body which facilitates the separation of the two primary layers of the skin. The effectiveness of this method of separating the epidermis as a sheet from the underlying dermis has been repeatedly checked by examining paraffin sections which were prepared from the tissue remaining after the removal of the epidermis. In every case the separation was found to be complete, for no trace of the epidermis was ever observed adherent to the dermis. The thin sheet of epidermis removed was stained as a whole mount with Ehrlich's haematoxylin, dehydrated, cleared in oil of wintergreen and xylol, and mounted in balsam on a slide with the proximal surface uppermost. The epidermis is so thin that all of the cells composing it can be examined with an oil-immersion objective, and mitotic figures are easily distinguished. Thus, mitosis counts can be made more readily and probably more accurately than on the usual serial sections. An additional reason for choosing the epidermis of the ear of the mouse as the tissue for study was that previous observations had been made in this laboratory (Cooper and Franklin (*16*)) on the diurnal rate of mitosis in the normal epidermis of the ear of the mouse. These observations furnished considerable data concerning the normal rate of cell division in the mouse epidermis.

Fifty-two strain A male mice were used in this study. They were approximately 2½ months of age at the beginning of the experiment and varied in weight from 14.5 to 27 gm. The mice were kept in a room in which the temperature was maintained at 78° F. by thermostatic control and were all fed the same diet, Rockland Farms mouse pellets. None of the treated mice died during the course of the experiment.

Both ears of each mouse were painted twice weekly with a 0.6-percent solution of methylcholanthrene (Eastman Kodak Co.) in benzene. The stock solution of methylcholanthrene was kept in the ice box when not in use to prevent evaporation of the benzene and consequent progressive concentration of methylcholanthrene. The carcinogen was applied with a single stroke

of a No. 3 camel's-hair brush, and all applications were made by 1 individual (Z. K. C.). Painting was begun on August 6, 1940, and 2 days following the first application of methylcholanthrene the ears were removed from 3 mice. Throughout the series the ears were removed 2 days after the previous painting. Thus the interval between the last application of the carcinogen and the removal of the ear was always the same. The right ear of each mouse was placed immediately in 1 percent acetic acid for the preparation of a total mount, and the left ear in Bouin's fixing fluid for the preparation of paraffin sections which would serve as a check on the degree of hyperplasia attained. At weekly intervals thereafter until November 7, 1940, the ears were removed from each of 3 mice. By this time all of the 11 remaining mice had developed either papillomas or carcinomas, and the epidermis of the ears was too thick to make the preparation of total mounts feasible. The last group of mice from which the ears were removed for study had received a total of 27 applications of methylcholanthrene.

The total mount of the epidermis from the inner surface of the right ear of each mouse was examined with an oil-immersion objective at a magnification of 1,350 diameters. Nuclei were counted, not cells, since the boundaries of cells are not easily determined. Fifteen thousand nuclei (nondividing and dividing) were enumerated in the epidermis of the inner surface of each ear. To facilitate the counts, a ruled square was inserted in the ocular of the microscope. Care was taken to space the counts in such a way that nuclei from representative regions of the ear were included. All of the nuclei which appeared in 1 square (usually about 75) were counted. Contiguous squares were then examined until 1,000 nuclei had been enumerated. On an average this required 15 oil-immersion fields. An area equivalent to that of a thousand nuclei was then skipped, and a second group of a thousand nuclei was counted. This procedure was repeated until the entire surface of the ear had been systematically traversed from side to side and from tip to base. Counts were not made in the necks of the hair follicles but only in the epidermis between follicles. For the sake of uniformity, all counts were made by the same person (H. R.).

Paraffin sections were prepared through the median longitudinal axis of the left ear and stained with haematoxylin and eosin. These sections served as a check for determining the degree of hyperplasia in the ears at the time of their removal.

Total mounts of the epidermis were prepared from the right ears of 5 untreated strain A male mice. Counts of the number of mitoses per 15,000 cells were made on each of these ears by the same method as that used on the ears of the mice which had been painted with methylcholanthrene. Thus, a figure was arrived at for the normal incidence of mitosis in the epidermis of strain A male mice.

RESULTS

Gross Findings

All of the mitotic counts were made upon animals which showed no gross evidence of papillomas or carcinomas. By the ninety-third day the ears had been removed from a total of 41 animals, 3 being used each week, with the exception of the last week when only 2 animals were left whose ears did not show evidence of neoplastic change. Of the remaining 11 animals, 8 had carcinomas on the ears, and 3 had papillomas. Some thickening of the ears of 10 of the mice had been noted in the third week of the experiment. Papillomas had appeared in 3 mice in the sixth week, and a carcinoma was first

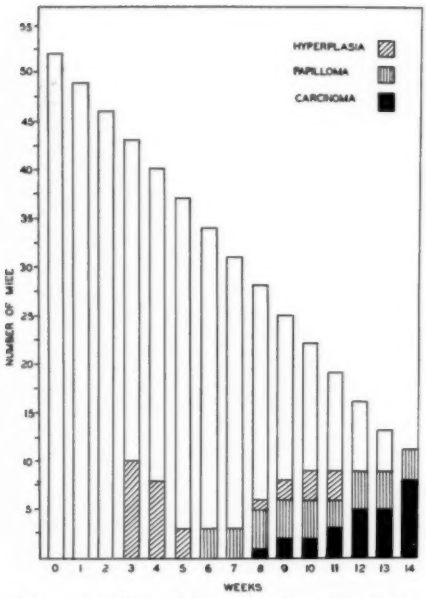

FIGURE 1.—Gross changes in the ears of mice painted with 0.6 percent methylcholanthrene in benzene.

noted in 1 mouse in the eighth week. Figure 1 shows graphically the gross changes observed in the ears of all of the 52 mice which were painted with methylcholanthrene.

The last 13 mice from which the ears were removed for study were then allowed to live out their lives without any subsequent paintings with the carcinogen. All died between the thirteenth and twenty-fourth week after the beginning of the experiment. Seven of them developed epidermoid carcinomas in the stumps of the ears, and 3 of them had sarcomas. These observations were confirmed by examination of microscopic sections. Such findings would indicate that the mice on which the mitotic counts were made were not cancer-resistant animals.

MITOSIS COUNTS ON TOTAL MOUNTS OF EPIDERMIS OF RIGHT EARS

In order to determine the normal rate of cell division in the epidermis of the ears of strain A male mice the number of mitoses per 15,000 nuclei was counted in the right ear of each of 5 untreated animals. These counts were 12, 17, 23, 19, and 14 mitoses per 15,000 nuclei, respectively, an average of 17 (table 1). This figure corresponds fairly closely to that obtained in previous experiments on untreated mice of the Old Buffalo Strain (Cooper and Franklin (*16*)). In these experiments similar counts were made upon the epidermis of the ears of 24 male mice of exactly the same age from which the ears were removed at hourly intervals for 24 consecutive hours. The average of those 24 counts was 21.7 mitoses per 15,000 nuclei.

In the present experimental group of mice the ears were removed from the first 3 individuals 2 days following the first painting with methylcholanthrene. The mitotic counts on these 3 ears were 2, 51, and 26 mitoses per 15,000 nuclei, thus showing considerable individual variation in the response to the first application of the carcinogen. The average number of mitoses per 15,000 nuclei for these 3 mice was 26.3. Even 1 painting apparently

TABLE 1.—*Mitoses per 15,000 nuclei, percentage of mitoses, and average number of nuclei per unit area in the epidermis of 5 untreated strain A male mice*

Mouse number	Mitoses per 15,000 nuclei	Percent of mitoses	Average number of nuclei per unit area
	Number		
1	12	0.08	52
2	17	.11	49
3	23	.15	52
4	19	.13	47
5	14	.09	49
Average	17	.11	49.8

produced an increase in the number of mitoses after 48 hours.

One week later, 9 days following the first application of methlycholanthrene, and after a total of 3 paintings, the ears were removed from 3 additional mice. The incidence of mitosis in the epidermis of these ears was 72, 83, and 79 mitoses per 15,000 nuclei, an average of 78 mitoses.

During the first 23 days of the experiment the number of mitoses continued to increase. In the 3 ears removed on the twenty-third day after the first painting, and after a total of 7 paintings, the counts were 171, 142, and 136, or an average of 149.7 mitoses per 15,000 nuclei. For the next 2 weeks the average count remained fairly constant. On the thirty-seventh day, after 11 paintings, there still were 152, 144, and 151 mitoses per 15,000 nuclei, or an average of 149.

From the thirty-seventh day to the sixty-fifth day, however, there was a steady decrease in the incidence of mitosis. On the sixty-fifth day, after 19 paintings, the number of mitoses per 15,000 nuclei in the ears of the three mice studied were 64, 85, and 69, an average of 72.7.

From the sixty-fifth day to the end of the experiment on the ninety-third day (after 27 paintings) there was a gradual increase in the number of mitotic divisions. In the last week of the experiment only 2 mice showed no gross evidence of papillomas or carcinomas. The final average count, 225 mitoses per 15,000 nuclei, is based, therefore, on 2 animals instead of 3.

The individual counts for each mouse and the percent of nuclei in mitosis are shown in table 2 and are presented graphically in figure 2.

Attempts were made to measure any changes that might occur in the number of nuclei per unit area during the course of the experiment, by counting the total number of nuclei included within the counter square in 5 sample areas of each ear. The square, when used in a ×15 ocular with a 2-mm. oil-immersion objective, covers an area measuring 57.5μ by 57.5μ. The average number of nuclei per square for the epidermis of the ears of the untreated mice was 49.8 (table 1). After 1 application of methylcholanthrene the number of nuclei per square increased to an average of 63. This increase in the number of nuclei per unit area may be accounted for by a decrease in nuclear

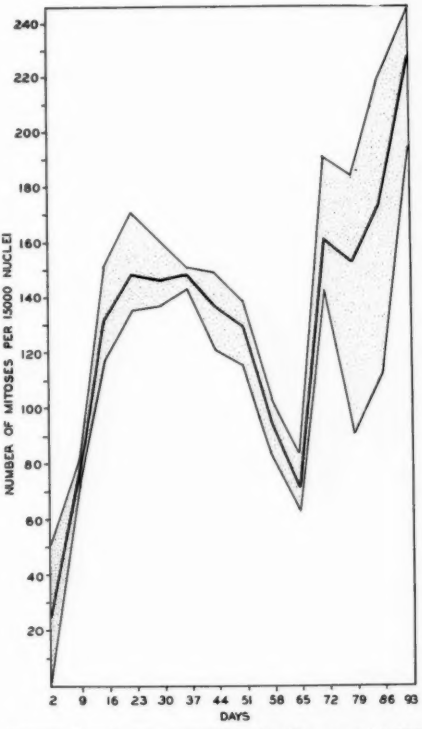

FIGURE 2.—Average number of mitoses per 15,000 nuclei in the epidermis of the ears of mice painted with 0.6 percent methylcholanthrene in benzene. The shaded area indicates the variation between lowest and highest counts.

TABLE 2.—*Mitoses per 15,000 nuclei, percentage of mitoses, and average number of nuclei per unit area in the epidermis of strain A male mice during the first 93 days of painting with methylcholanthrene*

Mouse No.	Time after first painting with methylcholanthrene	Paintings with methylcholanthrene	Mitoses per 15,000 nuclei	Percent of mitoses	Average Number of nuclei per unit area
	Days	*Number*	*Number*		
1			2	0.01	64
2	2	1	51	.34	67
3			26	.17	58
Average			26.3	.17	63.0
4			72	.48	68
5	9	3	83	.55	78
6			79	.53	69
Average			78.0	.52	71.7
7			129	.86	68
8	16	5	152	1.01	93
9			118	.79	76
Average			133.0	.89	79.0
10			171	1.14	87
11	23	7	142	.95	66
12			136	.91	72
Average			149.7	1.00	75.0
13			161	1.07	84
14	30	9	138	.92	99
15			143	.95	77
Average			147.3	.98	86.7
16			152	1.01	88
17	37	11	144	.96	80
18			151	1.00	65
Average			149.0	.99	77.7
19			142	.95	76
20	44	13	122	.81	84
21			150	1.00	77
Average			138.0	.92	79.0
22			117	.78	81
23	51	15	137	.91	78
24			140	.93	41
Average			131.3	.87	66.7
25			101	.67	73
26	58	17	103	.69	85
27			85	.57	69
Average			96.3	.64	75.7
28			64	.43	71
29	65	19	85	.57	68
30			69	.46	79
Average			72.7	.49	72.7
31			191	1.27	75
32	72	21	152	1.01	70
33			143	.95	69
Average			162.0	1.08	71.3
34			184	1.23	71
35	79	23	185	1.23	69
36			92	.61	66
Average			153.7	1.02	8.76
37			219	1.46	61
38	86	25	113	.75	76
39			191	1.27	71
Average			174.3	1.16	69.3
40	93	27	255	1.70	64
41			195	1.30	69
Average			225.0	1.50	66.5

size, or by an actual increase in the number of cells because of hyperplasia of the epidermis. Table 2 presents the average numbers of nuclei per unit area in the total mounts of the epidermis of the right ear of each experimental mouse, correlated with the number of applications of methylcholanthrene which the animal had received and time, after the first painting, at which the ear was removed for study. After the increase in the number of cells per unit area following the first painting, the variation was slight. There is no correlation apparent between the number of nuclei per unit area and the number of mitoses per 15,000 nuclei.

MICROSCOPIC SECTIONS OF THE LEFT EARS

The paraffin sections made from the central portion of the left ear of each mouse (which had also been treated with carcinogen) were examined for any changes which could be correlated with the mitotic indexes obtained on the total mounts of the right ears. The ears removed from the first 3 experimental mice 2 days after the first painting with methylcholanthrene showed very little change from the normal. The epidermis was only 2 to 3 cells in thickness. Sixteen days after the first application of methylcholanthrene (after 5 paintings) the epidermis of the ears had become definitely hyperplastic. Differ-

entiation of the epidermis into a basal layer, a squamous layer, and a granular layer had occurred; it was now from 4 to 6 cells in thickness. From the sixteenth to the sixty-fifth day of the experiment the degree of hyperplasia remained about the same and constituted a fairly uniform thickening of the entire epidermis. On the sixty-fifth day 1, on the seventy-second day 1, on the eighty-sixth day 2, and on the ninety-third day 1 of the mice showed a marked hyperplastic area at the base of the left ear. In these individuals, in limited areas, the epidermis had become about 15 cells thick and had lost its regular arrangement into layers. These areas were not definitely malignant but did conform to what has been termed "precancerous hyperplasia" (Paletta, Cowdry, and Lischer (*17*)). Similar areas appeared in the total mounts as localized thickenings and were observed in all 14 individuals studied from the sixty-fifth day to the end of the experiment. Since the response in the epidermis of the 2 ears of the same individual may be considered to be similar, the cross sections of the left ears served as a control on the total mounts.

No carcinomas developed during the experimental period, but in four of the individuals examined between the sixty-fifth and the ninety-third days of the experiment, a very marked hyperplasia was observed. This period, when in some of the individuals there was a marked increase in the degree of hyperplasia of the epidermis, coincides with the period in which there was the final marked increase in the number of mitoses following a period when the mitotic counts had been low.

DISCUSSION

The present observations indicate that methylcholanthrene produces an increase in mitotic activity in the epidermis which is apparent 48 hours after the first application of the carcinogen and which is progressive during the first 23 days of painting. A period of decreased frequency of mitosis which is marked and extends over several weeks follows. The percentage of cells in mitosis, however, at no time approaches that found in untreated epidermis. Following this interval when the number of cells undergoing division is decreased, there is a final rapid increased incidence of mitosis up to the time of the appearance of carcinoma when our observations ended. It would seem, therefore, that although methylcholanthrene produces an increase in cell division in the epidermis during the first weeks of painting, a period of decreased mitotic activity follows which persists up to the time when carcinoma appears in the series of mice. From the time when carcinoma begins to appear in the series, there is a definite increase in mitosis even in individuals who themselves show no gross or microscopic evidence of carcinoma.

During the first 65 days of the experiment, which covered the early period of increased frequency and the subsequent period of decreased incidence of mitosis, the individual response of each group of animals taken in successive weeks was much more uniform than it was during the end period of increased mitotic activity. This is shown in figure 2 in which the shaded area indicates the variation between lowest and highest counts while the solid line indicates the average number of mitoses throughout the experimental period.

During the first 65 days of the experiment no selection of animals whatever was made. All of the animals had been numbered consecutively at the beginning of the experiment, and the individuals were taken in numerical sequence through the sixty-fifth day. On the seventy-second day mouse No. 31, which was scheduled to be used that day, had an ulcerating tumor involving the proximal half of the right ear,

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which appeared, grossly, to be malignant. This tumor proved on microscopic section to be a squamous-cell carcinoma. The ear of this mouse was, of course, not taken for the preparation of a total mount since the precancerous period in the ear in this individual was already passed. From the seventy-second to the ninety-third day, similar individuals were eliminated from the experimental series, so that at the end of the experiment 11 animals were left all of whom showed gross evidence of neoplastic change. Thus, in some degree selection may have operated from the seventy-second day to the end of the experiment. This, however, is the terminal period of markedly increased mitotic activity. During the first 65 days of the experiment no selection was operative, so that this factor cannot be used to explain the initial rise in mitosis and the period of decreased mitotic activity which immediately follows it.

No adequate explanation can be given for the mid-period of lack of increase in mitosis. It would not appear, however, that the mice which showed the lowest mitotic counts were resistant animals who would never have developed carcinoma. Of the three mice used on the sixty-fifth day, on which the lowest number of mitoses in the mid-period were found, two were allowed to live out their normal lives after the removal of the ears. They received no additional applications of methylcholanthrene. At autopsy, however, both of these mice were found to have squamous-cell carcinomas of the stumps of the right ears.

In his work on tar cancer, Bang (*18*) called the time which elapsed between the first tarring and the occurrence of invasive growth the developmental period and divided it into a preparatory period which lasted for 2 or 3 months or until potential malignancy set in, and a latent period which ran from the end of the preparatory period up to the supervention of invasive growth. Champy and Vasiliu (*19*), also working on tar cancer in mice, studied the incidence of mitosis from the beginning of tarring up to 186 days. They counted the number of mitoses in 1,000 cells of the basal layer at various intervals. Their results were as follows:

	Mitoses
Normal skin	2–3
Skin after tarring—	
15 days	6
30 days	25
84 days	20
94 days	40
131 days	27
159 days	28
186 days (appearance of cancer)	42

Thus, in the development of tar-induced cancer as of methylcholanthrene-induced cancer, there seems to be an early period of augmented mitotic activity followed by an interval of decreased incidence of mitosis. A terminal period of increase in cell division occurs just preceding the actual appearance of carcinoma.

No definite correlation could be established between the variations in mitotic frequency and the changes in viscosity and in the nucleocytoplasmic ratio during the precancer period, which were reported by Cowdry and Paletta (*1*, *2*). The latter studies were made at longer intervals during the period of carcinogenesis and included observations on fully developed carcinomas, while the data on mitosis were collected at shorter intervals which extended only up to the time of the appearance of malignant change.

SUMMARY

A series of 52 mice were painted twice weekly with methylcholanthrene, and mitotic counts were made on total mounts of the epidermis of the ears of 3 mice each week for 14 weeks (except the last week when only 2 animals were available). At

this time the ears of all of the 11 remaining mice showed evidence of neoplastic change. Fifteen thousand nuclei were counted in each ear, and the number of mitoses encountered was recorded.

A marked increase in mitosis was found during the first 37 days of the experiment. From the thirty-seventh to the sixty-fifth day a decrease in the number of dividing cells occurred, which was in turn followed by a period of rapid increase in mitotic activity extending from the sixty-fifth to the ninety-third day.

During the first 65 days of the experiment the individual variation in the numbers of mitoses in the animals examined in any 1 week was small, but during the final period of stimulation of mitotic activity more variability in individual response was evident. This variability may in part be accounted for by the fact that during this period definite carcinoma was developing in some individuals in the series.

Since no animals in which gross evidence of neoplastic change was present were used in the experimental series, selection was perhaps operating in the choice of animals on which counts were made. However, neither selection nor the possibility that the animals used had a natural resistance to the carcinogenic action of methylcholanthrene can explain the mid-period of retardation of mitotic activity.

From this experiment it would seem that methylcholanthrene acts first as a stimulant to cell division and that this effect then wanes. When this latent period is passed and carcinoma is about to develop, a marked increase in mitotic activity again occurs.

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The Liver Catalase Activity of Tumor-Bearing Mice and the Effect of Spontaneous Regression and of Removal of Certain Tumors

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INTRODUCTION

It has been shown that the liver catalase activity of rats bearing transplants of either the hepatic tumor No. 31 (*1*) or the Jensen sarcoma were one-tenth to one-fifteenth as great as that of the livers of normal rats (*2-4*). This phenomenon, which suggests a systemic effect on the animal elicited by the presence of the tumor, was found to be reversible, for the complete excision of the hepatic tumor transplant produced a return of the liver catalase activity to the normal level within a relatively short time. Reinoculation of fresh tumor tissue into animals from which the first tumor had been excised caused again a lowering of the liver catalase. The latter again returned to normal after excision of the second transplant. Under the conditions used, namely, reinoculation with fresh tumor tissue 48 hours after extirpation of the first tumor, no resistance of the animal to the growth of the second transplant was apparent.

This study has now been extended to the investigation of the liver catalase activity of mice bearing various tumors. The study of this species offers a number of very interesting possibilities and opportunities since here one can work with several inbred strains. Not only is it possible, therefore, to observe the effect on the liver catalase activity of various tumors in known hybrids and in different strains of mice, but it is also feasible to observe the effect produced by a single, specific tumor in several inbred strains. A preliminary report of the former effect has already been made (*5*). A basis for the latter study has been provided by Andervont (*6-8*) who has shown that sarcoma 37 cutaneously inoculated into several inbred strains grows at different rates in certain strains. In some of the latter the tumor spontaneously regresses, leaving the animal resistant against the growth of a second inoculum of the same tumor. Andervont has further found (*7, 8*) that sarcoma 37 grows well when subcutaneously implanted in the tails of mice of susceptible strains, and that removal of the tail in which the tumor is growing confers the same resistance to growth of a second implantation in certain strains as does spontaneous regression of the tumor. These phenomena obviously provide many valuable approaches to a study of the possible systemic effects elicited by the presence of a tumor. The investigation of these and related phenomena, insofar as they affect the liver catalase activity of mice bearing tumors and of animals in which the tumor has either spontaneously regressed or from which the tumor has been physically removed, is reported in this paper.

EXPERIMENTAL PROCEDURE

The livers were obtained and prepared for the enzyme determinations exactly in the manner described for the same determinations on rats (*4*). Likewise, the technique for the catalase determinations was the same as that previously described (*4*).

The Effect of Residual Blood

A possible source of error in the determination of liver catalase activity lies in the fact that blood is always present in this organ, and the red cells of the blood contain catalase. In order to determine the magnitude of the contribution to the total catalase activity of the liver by the residual blood, samples of fresh mouse blood at the dilutions found in the liver extracts were studied for catalase activity. The dilutions were calculated as follows: If it is assumed that 10 percent of the total volume of the mouse liver consists of blood, certainly a very generous assumption, then the average mouse liver which weighs approximately 1.5 gm. will contain about 0.1 cc. of blood. The liver after grinding is extracted in about 4.5 cc. of water. Estimation of the concentration of total N in this extract yields as a rule 4–6 mg. N per cubic centimeter. The catalase determination are performed with extracts diluted to exactly 0.6 mg. N per cubic centimeter, so the original extract must be diluted from 7 to 10 times. The test extract, therefore, contains from 0.002–0.003 cc. of blood per cubic centimeter of extract which is the volume actually employed in the catalase determinations. We therefore investigated the catalase activity of normal mouse blood diluted from 1–1,000 to 3–1,000. The methods of determination and calculation were the same as those used for the liver extracts. The results, expressed as cubic centimeters of oxygen per second, were 0.003, 0.004, and 0.006 for dilutions of blood, respectively, at 1–1,000, 2–1,000, and 3–1,000.

The catalase activity, expressed similarly for normal mouse liver and for the lowest value found for the liver of a tumor-bearing mouse, was, respectively, 2.0 and 0.1. The contribution to the catalase activity of normal liver by 10 percent of residual blood is thus about 0.3 percent; to the liver whose activity is most lowered by the presence of a tumor borne by the mouse the contribution by 10 percent of residual blood is about 6 percent. The latter figure is well within the experimental error which is about 10 percent and the percentage variation between the activities of livers from animal to animal which is from 10 to 30 percent. We have assumed in the above calculation that the blood of normal animals has the same catalase activity as the blood from tumor-bearing animals. While our studies on the catalase activity of the blood of the latter animals is by no means extensive as yet, we have so far found little, if any, difference from that of the blood of normal animals. In any event we may safely ignore any contribution to the total catalase activity of the mouse livers which may be provided by the residual blood.

The Activity of Livers of Tumor-bearing Mice

The first extensive experiments were performed on the determination of the liver catalase activity of inbred strains of mice bearing various tumors.[1] For comparison the activity of the livers of normal mice of each strain was also determined.

[1] *The mice bearing the following tumors were very kindly provided by our colleagues in the National Cancer Institute: the transplanted gastric and pulmonary tumors by Dr. Stewart, the transplanted brain tumor by Dr. Shear, the transplanted hepatic tumor in C3H mice and the transplanted intestinal tumor by Dr. Thompson, the transplanted melanoma by Dr. Turner, the spontaneous mammary tumors in A mice by Dr. Heston, the transplanted lymphoma by Dr. Mider, and the transplanted malignant endothelioma by Dr. Edwards.*

TABLE 1.—*The liver catalase activity of mice bearing tumors*

[Temperature 27° C, 1.0 cc. of 29 percent H_2O_2 added to mixture of 1.0 cc. extract plus 5 cc. of M/5 phosphate buffer at pH 6.9]

Strain	Tumor	Age of tumor[1]	Concentration of N in extract	Catalase activity—O_2 evolved
		Days	*Mg. per cc.*	*Cc. per second.*
Normal C3H			0.6	1.9
			.3	.5
C3H	Transplanted hepatic	270	.6	1.9
			.3	.4
C3H	Spontaneous mammary.	18	.6	1.9
			.3	.4
C3H	do	35	.6	1.6
			.3	.2
C3H	do	60	.6	.7
			.3	.2
C3H	Transplanted adenocarcinoma of stomach.	21	.6	.3
			.3	.1
C3H	Transplanted brain	90	.6	.3
			.3	.09
C3H	Primary sarcoma induced by methylcholanthrene pellets.	14	.6	.5
			.3	.1
C3H	Transplanted malignant endothelioma.	30	.6	.1
			.3	.06
Normal A			.6	2.0
			.3	.6
A	Transplanted intestinal.	90	.6	1.9
			.3	.6
A	Spontaneous mammary.	1	.6	2.0
			.3	.6
A	do	35	.6	.6
			.3	.2
A	Transplanted pulmonary.	42	.6	.3
			.3	.1
A	Transplanted lymphoma.	9	.6	1.4
			.3	.3
		20	.6	.6
			.3	.1
A	Transplanted pulmonary tumor F.	30	.6	.5
			.3	.1
Normal C			.6	1.8
			.3	.4
C	Transplanted hemangioma.	60	.6	.7
			.3	.2
C	Transplanted sarcoma 37.	24	.6	.1
			.3	.06
Normal dilute brown.			.6	2.0
			.3	.5
Dilute brown	Transplanted melanoma.	40	.6	1.2
			.3	.3
Do	do	70	.6	.1
			.3	.06
Do	Transplanted sarcoma 37.	10	.6	.1
			.3	.06
Do	Transplanted CR 180	10	.6	.3
			.3	.1
Normal I			.6	2.0
			.3	.5
I	Transplanted hepatic	21	.6	.4
			.3	.1
I	Transplanted sarcoma 37.	10	.6	.4
			.3	.1
I	do	[35]	.6	1.9
			.3	.5
Normal C57 Black.			.6	1.0
			.3	.3
C57 Black	Transplanted sarcoma 37.	10	.6	.9
			.3	.3
Do	do	[35]	.6	.8
			.3	.2
Normal Y			.6	2.0
			.3	.6
Y	Transplanted sarcoma 37.	10	.6	.2
			.3	.1

[1] Age refers in the case of transplanted tumors to the time elapsed since implantation; in the case of spontaneous and of induced tumors to the time since the latter were palpable. The latter estimation of time is obviously only approximate. The brackets in this column refer to the fact that at this date the tumors had already spontaneously regressed and were no longer visible.

Several mice were used in each of these, as well as in subsequent determinations described below. The data are given in table 1.

The liver catalase activity of normal mice, with the notable exception of the C57 Black strain, is very nearly the same for all the strains studied and is slightly higher than that found in the case of rats (*4*). The activity of the livers of the C57 Black normal mice is just about half that of the other strains. This unexpected finding was confirmed by repeated studies on eight separate animals of this strain. The reason for the divergence from other strains in the values for the liver catalase of the C57 Black mice is not apparent. Figge and Strong (*9*) have found that the liver xanthine oxidase activity for the C3H strain is half that found in the JK strain.

With the exception of the transplanted sarcoma 37 in C57 Black mice, the transplanted hepatic tumor in C3H mice, and the transplanted intestinal tumor in A mice, the presence of all the other tumors studied caused a lowering of the liver catalase activity in the mice bearing the tumors, provided that the tumors had grown for a sufficient length of time. It is clear from table 1 that in the earlier stages of the growth of the spontaneous mammary tumors and of the transplanted lymphoma

and melanoma the liver catalase activity of the tumor-bearing animals was very nearly normal. Only after a much longer period of growth did the latter tumors produce a lowering of the liver catalase of the tumor-bearing mice. It is, of course, possible that if the transplanted hepatic and intestinal tumors had been allowed to grow further before the animals were killed the liver catalase activity might have been lowered. However, the age of the former tumor was 9 months, a far greater age than any other tumor had reached. Sarcoma 37 in C57 Black mice has been found to regress and disappear after about 3 weeks' growth (*8*). The failure to find any lowering of the liver catalase activity in C57 Black mice bearing sarcoma 37 for 10 days when the same tumor at the same stage of growth in C, I, dilute brown, and Y mice causes a marked lowering of the liver catalase activity, may in some way be related to the exceptional value of the liver catalase activity found in normal mice of the C57 Black strain. It is of great interest that sarcoma 37 in the tenth day of growth in I mice caused a distinct lowering in the liver catalase activity, but that by the thirty-fifth day, when the tumor had completely regressed, the liver catalase activity was back to the normal level.

It may be pointed out that the effect of lowering the liver catalase of tumor-bearing mice can be produced by spontaneous and induced as well as by transplanted tumors. The effect produced by the spontaneous mammary tumors in A or C3H mice is considerably less than that produced in a much shorter period by sarcoma 37 in dilute brown mice. The true age of the spontaneous and induced tumors described in table 1 is very much greater than that given. The latter dates from the day that the tumors were palpable, but it is of course true that the tumors may have begun to develop some weeks prior to this date.

The Effect of the Progressive Growth of Lymphoma No. 72942 on the Liver Catalase Activity

The discovery that some transplanted tumors in the early stages of growth do not affect the liver catalase activity whereas in the later stages of growth the enzymatic activity of the liver is lowered led us to investigate the effect produced by lymphoma No. 72942 during various times subsequent to implantation of the tumor. The data are given in table 2.

It is clear from table 2 that with the progressive growth of the tumor there was a proportional drop in the liver catalase activity of the tumor-bearing animals. The activity values appeared to approach a constant value just before the death of the animals bearing this tumor, which occurred in a little more than 3 weeks after implantation of the tumor.

Sarcoma 37 in Five Inbred Strains

Andervont (*7*, *8*) showed that when sarcoma 37 was inoculated cutaneously in mice of the dilute brown and Y strains the tumor grew rapidly and killed the hosts.

Table 2.—*Liver catalase activity of mice bearing transplanted lymphoma No. 72942 in A mice*

Age of tumor [1] (days)	Concentration of N in extract	Catalase activity—O_2 evolved
	Milligrams per cubic centimeter	*Cubic centimeters per second*
0 (normal A mice)	0.6	2.0
	.3	.6
2	.6	1.6
	.3	.4
6	.6	1.5
	.3	.3
9	.6	1.4
	.3	.3
12	.6	1.0
	.3	.3
15	.6	.9
	.3	.2
18	.6	.7
	.3	.2
19	.6	.6
	.3	.1
20	.6	.6
	.3	.1

[1] Age refers to time elapsed since implantation.

TABLE 3.—*Liver catalase activity of mice carrying cutaneous implants of sarcoma 37 and the effect of spontaneous regression of the tumor*

Age of tumor [1] (days)	Concentration of N in extract	Catalase activity in strain—O_2 evolved				
		Dilute brown	C57 Black	Y	I	C
	Mg. per cubic centimeter	*Cc. per second*	*Cc. per second*	*Cc. per second*	*Cc. per second*	*Cc. per second*
0 (normal mice)	0.6	2.0	1.0	2.0	2.0	1.8
	.3	.5	.3	.6	.5	.4
1	.6	1.8	.....	.....	.....	.....
	.3	.3	.....	.....	.....	.....
2	.6	1.1	.....	.....	.....	.....
	.3	.2	.....	.....	.....	.....
3	.6	1.0	.....	.....	.....	.....
	.3	.2	.....	.....	.....	.....
4	.6	1.1	.....	.....	.....	.....
	.3	.2	.....	.....	.....	.....
5	.6	.8	.....	.....	.....	.....
	.3	.1	.....	.....	.....	.....
7	.6	.2	.7	.4	1.0	.4
	.3	.08	.2	.2	.3	.1
10	.6	.1	.9	.2	.4	.3
	.3	.06	.3	.1	.1	.1
14	.6	.1	.6	.1	.7	.2
	.3	.06	.1	.05	.1	.09
17	.6	.....	.7	.....	1.3	.2
	.3	.....	.2	.....	.3	.1
21 [2]	.6	.....	.7	.....	1.3	.1
	.3	.....	.2	.....	.3	.08
24	.6	.....	.6	.....	1.7	.1
	.3	.....	.1	.....	.4	.06
35	.6	.....	.8	.....	1.9	.....
	.3	.....	.2	.....	.5	.....

[1] Age refers to time elapsed since implantation.
[2] At this stage the tumors in the I and the C57 Black mice had regressed.

In C57 Black mice the tumor grew rapidly for about 3 weeks and then regressed completely in virtually all the animals. In I mice the tumor grew more slowly up to about 2 weeks and then regressed completely. In strain C mice the tumor grew slowly but progressively, ultimately killing the animals.

Mice of each of the five strains were cutaneously inoculated with sarcoma 37, and from time to time a few mice from each group were killed and the liver catalase activity was determined. The results are given in table 3.

Dilute brown strain

The liver catalase activity dropped to about half the normal value at about the second day after implantation. The activity dropped sharply between the fifth and seventh days, finally attaining a value of about one-twentieth the normal from the tenth to the fourteenth day after implantation. The animals as a rule did not live much longer than the latter period.

Y strain

These animals followed much the same course as the dilute brown mice, the liver catalase activity dropping to about one-twentieth the normal value 14 days after implantation of the tumor.

C strain

The tumor in these mice grew somewhat slower than in the above two strains, but the rate of fall in the liver catalase activity was not much slower than in those strains. The low value of the activity in the C strain simply persisted for a longer period of time until the host animal ultimately died.

I strain

The liver catalase activity dropped to about half the normal value at 7 days following implantation. At this stage, the liver catalase activity value was still considerably greater than the values shown by the foregoing three strains. By the tenth day, the liver catalase activity in the I mice had dropped still further, to about one-fifth the normal value, which was still higher than the corresponding activities of the dilute brown and Y mice at the same period following inoculation. By the fourteenth day a rise in the liver catalase activity of the I mice was apparent. This rise coincided with definite evidence of spontaneous regression of the tumor. The catalase activity continued to rise in the livers of the I mice, and at the stage of 21 days following implantation, when the tumor had completely regressed, the liver catalase activity was nearly 70 percent of the normal value. The latter value was definitely reached at 4–5 weeks after the date

of implantation of the tumor. The restoration of the normal activity of the liver proceeded approximately parallel to the regression of the tumor in the I strain. The fact that at no time in the period of growth of the tumor, i. e., up to 10 days following implantation, did the liver catalase activity ever reach so low a value as in the dilute brown, C, or Y strains, may be tentatively attributed to the supervening of the process of regression on the effect produced by the tumor on the liver catalase.

C57 Black strain

These mice, to begin with, have a normal liver catalase activity about half that of the other strains studied. There appears, furthermore, to be relatively little change in the liver catalase activity during the growth and the regression of the tumor. These facts set off the properties of the livers of this strain in marked contrast with the behavior of those of the other strains studied.

The Effect of Reimplantation of Sarcoma 37

It has been shown by Andervont (*7*, *8*) that I and C57 Black strains in which sarcoma 37 had completely regressed were immune to the growth of a second, subcutaneous inoculation with this tumor. In order to observe whether this second inoculation into immune I mice was accompanied by any change in the liver catalase activity, mice of this strain were inoculated with sarcoma 37, 35 days after the first implantation had occurred and about 2 weeks after this first implant had completely regressed. The second inoculum failed to grow in these mice, thus confirming the earlier work, and the liver catalase activity of these mice remained at a normal level at 1 to 2 weeks following the second inoculation. The results are shown in table 4.

TABLE 4.—*Effect of implantation, spontaneous regression, and reimplantation of sarcoma 37 on the liver catalase of strain I mice*

Description of mice	Age of tumor[1]	Concentration of N in extract	Catalase activity—O_2 evolved
	Days	*Milligrams per cubic centimeter*	*Cubic centimeters per second*
Normal I mice		0.6	2.0
		.3	.5
I mice carrying cutaneous implant of sarcoma 37.	7	.6	1.0
		.3	.3
Do	10	.6	.4
		.3	.1
Do	14	.6	.7
		.3	.1
Do	17	.6	1.3
		.3	.3
Do	[2] 21	.6	1.3
		.3	.3
Do	24	.6	1.7
		.3	.4
Do	35	.6	1.9
		.3	.5
Mice same as those described immediately above, reinoculated with sarcoma 37.	7	.6	1.9
		.3	.5
Do	14	.6	2.0
		.3	.5

[1] Age refers to time elapsed since implantation of tumor.
[2] At this stage the tumors had regressed.

Part of the results in this table are taken from table 3.

Sarcoma 37 in the Tails of Inbred Strains and the Effect of Removal of the Tails

It has been shown by Greenstein, Jenrette, and White (*3*, *4*) that the lowered liver catalase activity of a tumor-bearing rat could be restored to the normal level by complete excision of the tumor. In the present communication (tables 3 and 4) it is shown that the spontaneous regression of sarcoma 37 in strain I mice results in a restoration of the liver catalase activity to the normal level. Although this latter finding is of considerable interest, it is difficult to correlate *exactly* the restoration of the liver catalase with the regression of the tumor since the dates when the regression begins and when it finally ends can be only approximately determined.

It was considered desirable, therefore,

TABLE 5.—*Liver catalase activity of mice with sarcoma 37 transplanted subcutaneously into the tails and the effect of removing the latter* [1]

Age of tumor [2] (days)	Time elapsed since removal of tails	Concentration of N in extract	Catalase activity of strain—O_2 evolved				
			Dilute brown	C57 Black	Y	I	C
	Days	*Mg. per cubic centimeter*	*Cc. per second*	*Cc. per second*	*Cc. per second*	*Cc. per second*	*Cc. per second*
0 (normal mice)		0.6	2.0	1.0	2.0	2.0	1.8
		.3	.5	.3	.6	.6	.4
10		.6	.9	.8	.5	1.3	1.3
		.3	.2	.2	.1	.2	.2
16		.6	.6	.7	.4	.9	.8
		.3	.1	.2	.1	.1	.2
	1	.6	.4	.7	.3	.6	.6
		.3	.09	.2	.08	.2	.2
	2	.6	1.7	.6		1.7	1.7
		.3	.4	.2		.3	.3
	3	.6	2.0	.6		1.8	1.8
		.3	.5	.2		.4	.4
	4	.6	1.9	.6		2.0	1.9
		.3	.4	.2		.5	.4
	5	.6	2.0	.7			1.9
		.3	.5	.2			.4
	7	.6	1.8	.7			
		.3	.4	.2			
	8	.6	1.8	.7			
		.3	.4	.2			
	9	.6	2.0	.6			
		.3	.5	.2			

[1] The liver catalase of normal mice of the dilute brown and C57 Black strains whose tails were removed did not vary appreciably from the values found for the normal mice of these strains.
[2] Age refers to time elapsed since implantation of the tumor.

to follow the liver catalase activity from a time when the tumor at a single step is completely removed from the animal. For this purpose, the finding by Andervont (*6*, *7*) that sarcoma 37 grows progressively in the tails of mice of various strains was employed.[2] Mice of the dilute brown, C57 Black, Y, I, and C strains were inoculated caudally with this tumor (*7*). The latter grew progressively in each strain up to 16 days, although at a slower rate than when the tumor was implanted cutaneously. On the sixteenth day after inoculation the tails were amputated. The liver catalase activity of the various strains with sarcoma 37 growing in the tail, and the effect on the enzymatic activity of the livers of mice from which the tails were amputated are shown in table 5.

A comparison of the data in tables 3 and 5 reveals that the liver catalase activity of the dilute brown, I, Y, and C strains carrying sarcoma 37 in either the skin or the tails was considerably lower than normal. The activity at the same age of the tumor in the former site was very much less than that in the latter site. The tumor in the skin grew faster than in the tails, and it is possible that this is one explanation for the difference found in the liver catalase values. The activity of the livers of mice carrying the sarcoma in the tail also decreased with age of the tumor (table 5). Mice of the C57 Black strain again proved to be an exception to the above findings, for little change in the liver catalase activity was evident.

The tails bearing the transplanted sarcoma 37 were amputated on the sixteenth day following implantation of the tumor.[3] One day after this operation the liver catalase values actually decreased further in all

[2] *Andervont* (*7*) *found that amputation of the tails then confers an immunity against the growth of a second inoculum of the same tumor in the groin in about 60 percent of the animals studied.*

[3] *Within several days after this date the tumors in the tail of the I and C57 Black mice regressed.*

the strains studied except the C57 Black. Two days after operation the liver catalase activity of the dilute brown, I, and C mice returned to nearly the normal value, and by the third day the liver catalase activity of these strains was completely normal. The behavior of the mouse livers for the first day after removal of sarcoma 37 was in marked contrast with the properties of rat livers for the first day following extirpation of the hepatic tumor. In the latter case there was a sharp rise to nearly the normal value within this relatively short space of time. The return of the mouse liver catalase activity to the normal value was slower than that of the rat by about a day. This particular comparison is limited only to the case described since it must be remembered that the effect of two quite different tumors is being considered, and moreover the manner of removing the tumors from each of the species is different.

Of perhaps more general applicability is the finding that although the liver catalase activity may be lowered in rats and in mice carrying a tumor in different sites, complete removal of the tumor effects within a relatively short space of time a return of the liver catalase value to the normal level. The behavior of the C57 Black strain provides an exception to the effect of sarcoma 37, but it must be remembered that the value for the normal liver catalase of this strain appears to be anomalous and falls well outside the range found in all other strains studied.

Catalase Activity in Hybrid Mice

The transplanted hepatic tumor No. 1 (*1*) which was first grown in mice of the I strain can also be successfully propagated in I × dilute brown and I × C3H hybrids. The catalase activity of the transplanted hepatic tumor in the I × dilute brown hybrids has been reported by Greenstein, Jenrette, Mider, and Andervont (*5*). In order to observe whether the genetic constitution of the host mouse exerts any influence on the tumor and the latter in turn on the liver of the host mouse, the catalase activity of transplanted hepatic tumor No. 1 and the livers of animals bearing this tumor was investigated. The results on the normal hybrids are included for comparison. The hybrids were in the F_1 generation. The data are given in table 6.

It is clear from the results in table 6 that the catalase activity of the tumor studied and the activity of the livers of normal and of tumor-bearing animals were the same in the inbred and in the hybrid mice. The activity of the livers of the tumor-bearing mice was considerably lower than that of normal mice and was very nearly the same as that of the transplanted hepatic tumor. The catalase activity of this mouse tumor was very much greater than that of

Table 6.—*Liver and transplanted hepatic tumor catalase activity in hybrid mice (F_1 generation)*

Description of tumor	Age of tumor[1]	Tumor		Liver	
		Concentration of N in extract	Catalase activity —O_2 evolved	Concentration of N in extract	Catalase activity —O_2 evolved
	Days	*Mg. per cubic centimeter*	*Cc. per second*	*Mg. per cubic centimeter*	*Cc. per second*
Normal I mice				0.6	2.0
				.3	.5
I mice carrying transplanted hepatic tumor.	21	3.0	2.9	.6	.4
		1.5	1.7	.3	.1
		.6	.4		
Normal I × dilute brown mice.				.6	2.1
				.3	.5
I × dilute brown mice carrying transplanted hepatic tumor.	21	3.0	2.9	.6	.4
		1.5	1.5	.3	.1
		.6	.4		
Normal I × C3H mice				.6	2.0
				.3	.5
I × C3H mice carrying transplanted hepatic tumor.	21	3.0	3.0	.6	.4
		1.5	1.6	.3	.1
		.6	.4		

[1] Age refers to time elapsed since implantation.

transplanted hepatic tumor No. 31 in the rat (*2*, *5*).[4]

DISCUSSION

The results presented in this and earlier papers (*4*, *5*) indicate that the liver catalase system of rats and of mice, with few exceptions, is depressed by the presence of a tumor in these animals. That this effect is readily reversible is attested by the fact that removal of the tumor or a regression of the tumor is followed in a comparatively short time by a restoration of the liver catalase value to the normal level. Furthermore, histologic examination of the livers of tumor-bearing rats in which the liver catalase activity is about one-fifteenth that of normal, shows no apparent deviation from that of the normal organ (*4*). Nevertheless, a reduction of enzymatic activity from one-tenth to one-twentieth of its normal value indicates that a rather profound effect has occurred and suggests that some toxic material given off during the growth of the tumor travels to the liver and affects the catalase system with the results shown (*4*). The well-known cachexia of cancer must have some biochemical basis, and it may be that this phenomenon constitutes a contributing factor. It must be emphasized, however, that not all the liver enzyme systems of tumor-bearing animals are affected in this way. Thus, in the rat, xanthine dehydrogenase, depolymerase, and amylase are unaffected by the presence of the transplanted hepatic tumor (*2*). In the mouse, these same enzymes, together with arginase, also remain unaffected. It would appear so far that the effect of a tumor in an animal is most marked on the liver catalase. However, the study of other enzyme systems is obviously needed. Catalase is an iron-porphyrin system, and whether other iron-containing enzyme systems of the liver will be affected in the same way remains for further investigation.

The lowering of the liver catalase activity appears to be caused by spontaneous and induced as well as by transplanted tumors. The effect on the liver catalase is in a general way proportional to the rate of growth of the tumor, although no exact relationship has as yet been developed. Transplanted sarcoma 37 which grows very rapidly when subcutaneously inoculated into dilute brown and Y strain mice causes a marked lowering of the liver catalase activity of the tumor-bearing animals after 1 week of growth. The same tumor inoculated into the tail, where it grows more slowly, produces a much smaller effect on the liver catalase activity. Again, transplanted hepatic tumor No. 1, which has a high rate of growth in I mice, produces a marked decrease in 3 weeks in the liver catalase activity of these mice. The slow-growing hepatic tumor in C3H mice produces, even after 9 months, no effect on the liver catalase. The liver catalase of mice bearing the transplanted melanoma at 40 and at 70 days after implantation was reduced in activity to the same degree as shown by the liver catalase of mice of the same strain bearing sarcoma 37 for 2 and for 10 days, respectively. It cannot be definitely stated that sarcoma 37 exerts intrinsically a more powerful effect on the liver catalase than does the melanoma. As a statement of fact, all that can be said is that the former tumor grows faster than the latter.

If the cause of this effect on the liver catalase be tentatively ascribed to a toxic material elaborated by the cells of the tumor and thence circulated to the liver, then it would appear that a rapidly grow-

[4] *In the course of preliminary investigations on the liver catalase activity of other species, it was noted that the activity of normal guinea-pig liver is about 10 to 15 times greater than that of the mouse. In contrast with this, the activity of normal rabbit liver is about one-tenth that of the mouse.*

ing tumor producing this material in a short space of time would cause the liver to accumulate enough of it to produce the demonstrated lowering of activity. The liver in such a case would be relatively quickly affected by the toxic material. On the other hand, the liver in a slow-growing tumor would not only accumulate this toxic material slowly but would probably also have time to remove it in part. The only exception noted as yet of the failure of a rapidly growing tumor to affect appreciably the liver catalase is sarcoma 37 transplanted into mice of the C57 Black strain. This case apparently occupies an exceptional position, in view of the very low value found for the catalase activity of the livers of normal mice of this strain.

It is, of course, possible that the rate of growth may not be entirely responsible for the difference in the rate at which the effect on the liver catalase becomes manifest and that specific effects produced by different substances in each tumor may contribute their share. The liver catalase activity of both rats and mice, with few exceptions, is lowered early or late by the presence of a wide variety of tumors. What the nature of the substance which acts on the liver catalase could be is, of course, unknown at present, but it seems improbable, even if not altogether impossible, that it would be different for each tumor and yet produce an identical effect. However, we have at present no information on this problem and little preference in hypothesis is even possible. It is obvious that this problem is only at a beginning and that a good deal of further work is necessary.

SUMMARY

The liver catalase activity of mice bearing the following tumors was determined and found to be much lower than normal: in C3H mice, transplanted gastric adenocarcinoma, brain, methylcholanthrene-induced sarcoma, endothelioma, and spontaneous mammary; in A mice, transplanted pulmonary, lymphoma, and spontaneous mammary; in C mice, transplanted sarcoma 37 and hemangioma; in dilute brown mice, transplanted sarcoma 37, CR 180, and melanoma; in I mice, transplanted hepatic and sarcoma 37; in Y mice, transplanted sarcoma 37. The slower growing tumors did not show this effect until late in their development. The effect of the tumors on decreasing the liver catalase was found to be progressive with the growth of the tumors. The tumors which did not show this effect were the transplanted hepatic tumor in C3H mice up to 9 months after implantation, the transplanted intestinal tumor in A mice up to 3 months after implantation, and the transplanted sarcoma 37 in C57 Black mice. The value for the normal liver catalase of the latter strain was found to be anomalous.

Sarcoma 37 was inoculated cutaneously in five inbred strains: dilute brown and Y, in which it grew progressively and quickly; C, in which it grew progressively and more slowly than in the foregoing; I, where it grew slowly and then completely regressed; and C57 Black, where it grew rapidly and then completely regressed. The liver catalase activity was studied in each of these strains, and it was found that this activity decreased progressively with the growth of the tumor in the first three strains, decreased progressively with the growth of the tumor and then increased progressively with the regression of the tumor in the I strain, and finally did not appreciably alter at all in either direction during the growth and the regression of the tumor in the C57 Black strain. The I mice after regression of the tumor were resistant to a growth of a second inoculum of the same tumor, and subsequent to the

second inoculation the liver catalase of these mice remained at the normal level.

Mice of these five strains were inoculated in the tail with sarcoma 37. The tumors grew more slowly than in the skin and the decrease in the liver catalase activity at corresponding times was less in the caudally inoculated than in the cutaneously inoculated mice. At 16 days after implantation the tails were amputated. The liver catalase activity decreased still further at 1 day following this operation but by the second day was nearly normal, and by the third day and subsequently thereafter was at a completely normal level. Again the mice of the C57 Black strain provided an exception, for the liver catalase activity did not vary appreciably from the normal value for this strain either during the growth of the tumor or after removal of the latter. The return of the liver catalase value to the normal level following removal of the tumor is similar to the results obtained previously using rats bearing subcutaneously implanted hepatic tumor No. 31.

The catalase activity of a transplanted hepatic tumor grown in I mice and in the F_1 hybrids of this strain with C3H and with dilute brown mice was found to be the same. The activity of the livers of the tumor-bearing mice was the same in the hybrids as in the inbred strain, and while practically equal to that of the tumor, was considerably lower than that found in the livers of the corresponding normal animals.

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A Method of Evaluating Thymonucleodepolymerase Activity in Normal and Tumor Tissues

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The results obtained on incubating milk, sera, and a wide variety of normal and of tumor tissue extracts with solutions of sodium thymonucleate were reported previously (*1–4*). The reaction between each tissue preparation and the substrate was followed by measuring the viscosity of the mixture at an external pressure of 16.0 cm. of water over a 4-hour period of incubation in the viscometer at 30° C.

When the tissue preparations were added in equal volume to a 1.0-percent solution of thymonucleate, the value of the relative viscosity of the mixture immediately after mixing was far below that of a 0.5-percent solution of thymonucleate in water under the same conditions. Since the value of the relative viscosity of the latter solution is about 50 times that of any one of the tissue extracts employed, we may consider the change in viscosity brought about by mixing tissue extract and substrate solution as being due to a change in the physical properties of the thymonucleate, neglecting any possible changes in the components of the tissue extract as being negligible by comparison. It has been demonstrated that this rapid change in the physical properties of sodium thymonucleate is due wholly or in part to the presence of proteins, salts, and possibly protein split-products in the tissue extracts employed (*1*). Thus, solutions of various salts, amino acids, and crystalline proteins such as egg albumin and horse serum albumin when added to thymonucleate solutions all produce an instantaneous drop in the viscosity, the extent of the drop depending upon concentration, and in the case of salts and amino acids on the nature of their specific chemical groupings. Proteins are by far the most effective agents in this respect.[1]

Beyond this initial effect, all of the tissue extracts studied behave subsequently during the incubation period in either one of two ways: (1) Either the initial drop in the value of the viscosity is maintained practically constant with time; or (2) this initial value of the viscosity of the mixture is further lowered gradually with time, approaching asymptotically that of the viscosity of the tissue extract itself. The former property, described under (1), is characteristic of solutions of pure proteins, salts, amino acids, and the milk and sera of the human, cow, goat, and horse. The effect described under (2) is produced by the milk and sera of the mouse, rat, guinea pig, rabbit, and dog, and by extracts of various tumors and normal control tissues. We recently found that crystalline ribonuclease from the pancreas follows the first, and that extracts of plant seeds follow the second (*2*). We interpreted the second effect as being due to the presence of a thermolabile enzyme system in tissues which depolymerizes thymonucleic acid (*1*). To this enzyme we gave

[1] *If the protein solutions are heated at 60°–65° for many hours and then cooled and mixed with the thymonucleate solution, this drop occurs to only a negligible extent, thus showing that the effect of the proteins on the physical properties of sodium thymonucleate depends upon the native, unaltered state of the former* (1).

the name thymonucleodepolymerase. The greater the activity of this enzyme in any one tissue, the greater was the extent of the initial drop in viscosity after mixing enzyme and substrate. Superimposed on the drop due to the tissue proteins, etc., is the effect of the enzyme.

The activity of this enzyme was represented graphically heretofore by plotting the relative viscosity of the reaction mixtures against the time of incubation (*1–4*). The degree of activity of the enzyme in the particular source studied could be visually estimated by observing the rate at which the viscosity of the mixture approached the lower limiting value of the extract itself. An examination of the figures in the previous reports (*1-4*) shows the great variation of the activity in the different tissue extracts studied. Since such a method of visual estimation does not lend itself to numerical expression, it was considered desirable to set up criteria whereby the activity of the enzyme in the various tissue extracts could be evaluated and expressed in quantitative terms. The method adopted is as follows:

The measure of thymonucleodepolymerase activity is defined as follows:

Activity = $[\eta/\eta_0$ 0.5 percent thymonucleate − (η/η_0 1.0 percent thymonucleate + equal volume tissue extract)] − [η/η_0 0.5 percent thymonucleate − (η/η_0 1 percent thymonucleate + equal volume horse serum albumin at pH 7.0 and same total N as tissue extract)]. η/η_0 = relative viscosity. These differences are calculated at an incubation temperature of 30° C., at an incubation period of 30 minutes, and at a pH of 7.0. The second difference described above, involving horse-serum-albumin concentrations of 4.92, 2.46, 1.23, and 0.41 mg. of total N per cubic centimeter is, respectively, 31.09, 29.37, 26.91,

TABLE 1.—*The thymonucleodepolymerase activity*[1] *of various normal and tumor tissue and tissue extracts*

Source material	Total N in tissue extract	Thymonucleodepolymerase activity
	Milligrams per cubic centimeter	
Milk:		
Rat	4.92	16.00
	2.46	12.36
Mouse	4.92	8.07
	2.46	4.61
Rabbit	4.92	7.00
	2.46	4.70
Guinea pig	4.92	7.31
	2.46	4.36
Sera:		
Mouse (normal)	4.92	7.57
Mouse (spontaneous mammary carcinoma)	4.92	7.58
Rabbit	4.92	6.94
Guinea pig	4.92	5.57
Dog	4.92	17.14
Comparison studies:		
Normal mouse livers	1.23	14.32
Transplanted hepatic tumors in C3H mice	1.23	21.32
	.41	16.41
Transplanted hepatic tumors in I × dilute brown mice	1.23	11.07
Normal rat livers	1.23	6.61
Regenerating rat livers (2 days after removal of lobes)	1.23	6.26
Transplanted hepatic tumor No. 31 in rats	1.23	5.26
Normal rat spleens	1.23	1.74
Spleens of rats carrying transplanted hepatic tumor No. 31	1.23	1.74
Normal mouse lymph nodes	1.23	[2]
	.41	24.91
Normal mouse bone marrow	1.23	7.32
	.41	3.57
Transplanted lymphoma No. 72942	1.23	3.91
	.41	1.69
Hyperplastic breast in mice	.41	5.01
Spontaneous mammary tumors in C3H mice	1.23	19.46
	.41	14.00
Normal intestine of mice—first third	1.23	14.74
	.41	8.49
Transplanted intestinal tumor in mice	1.23	14.41
	.41	8.52
Normal mouse spleens	1.23	14.74
	.41	8.49
Cattle tissues:		
Beef pancreas	1.23	[2]
	.41	24.16
Calf thymus	1.23	2.32
Plant seeds:		
Sunflower	1.23	4.32
Lima bean	1.23	5.16
Pumpkin	1.23	6.41
Corn	1.23	6.24
Wheat	1.23	4.32

[1] Definition of activity given in text.
[2] Too great to measure.

and 24.41.[2] The enzyme activity will obviously be described in absolute numbers. From the data collected on all of the tissues studied (*1–4*), the enzymatic activity has been evaluated by the definition given above and the calculations are given in table 1.

Extensive comparisons of enzymatic activity in the various tissues studied have been made in the previous reports (*1–4*), and it is only necessary here to summarize briefly some of the more outstanding points up to the present: (1) The most active tissues studied appear to be the lymph nodes and the pancreas; (2) mouse tissues are more active than the corresponding tissues in the rat; (3) the milk and sera of rodents and the serum of the dog appear to contain the enzyme; (4) dog serum and rat milk are the most active of the body fluids studied; (5) the rat hepatic tumor is somewhat weaker than normal rat liver; a marked difference is revealed in the hepatic tumors in two different strains of mice whereby one is more active, the other less active than normal mouse liver; (6) whereas the spontaneous mammary tumor is much more active than hyperplastic mammary tissue, the activity of the lymphoma is very much less than that of normal lymph nodes; on the other hand the activity of the normal intestine and of the transplanted intestinal tumor is very nearly the same; (7) the sera of normal and of tumor-bearing mice are equal in activity; likewise the spleens of normal and of tumor-bearing rats are equal in activity. A further point of general biologic interest, not heretofore made, is that the thymonucleodepolymerase activity of the plant seed extracts is equal to and even greater than that of a number of the animal tissues studied, thus lending weight to the contemporary view that no distinctions should be drawn between the nucleic acids of plants and of animals.

[2] *The second difference in the equation defining the enzyme activity, which is given in terms of numbers related to the viscosity of serum albumin-nucleic acid mixtures, represents an arbitrary base line for the estimation of this activity. The assumption is tacitly made that at zero time, i. e., the time of mixing any extract solution with thymonucleate, the viscosity of this mixture will be the same as that of a mixture of thymonucleate with albumin of the same N concentration as the extract. In this way, the effect of the protein in the extract, as distinct from the effect of the enzyme, is evaluated. Obviously this is an arbitrary procedure, but in view of the physical limitations imposed by the viscosity method and the difficulty of estimating the inert protein effect apart from the enzyme effect in most tissues, this method is offered as being the most convenient at the present time for the estimation of depolymerase activity.*

SUMMARY

A method is described whereby the thymonucleodepolymerase activity of various tissues may be defined in absolute numerical terms. The results of the study of this enzyme to date are briefly summarized.

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Studies in Carcinogenesis. XVII. Local Effect of Repeated Application of 3,4-Benzpyrene and of Human Smegma to the Vagina and Cervix of Mice

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This paper reports the production of vaginal tumors in mice following repeated intravaginal application of a preparation containing 5 percent of 3,4-benzpyrene in cholesterol. The experiment was part of a preliminary exploration of the hypothesis that smegma may possess carcinogenic properties; it was carried out to ascertain whether the tissue lining the vaginal tract of the mouse would be responsive to the tumor-producing action of low doses of a known carcinogen. While tumors were obtained with benzpyrene, the experiments carried out with smegma gave negative results.

EXPERIMENTAL PROCEDURE

The smegma employed was obtained from men, elderly in most instances, who were confined in institutions.[1] After retraction of the foreskin, the smegma was scraped into a flask containing a 0.9-percent solution of sodium chloride and stored in the refrigerator. The benzpyrene employed had a melting point of 178°–179° corr. The experimental animals were young strain A mice obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The ration consisted of Purina dog chow and tap water, offered freely at all times.

[1] *The smegma was obtained through the courtesy of the Boston State Hospital, and of Dr. M. L. Dressler, of the Cambridge Home for the Aged.*

SUBCUTANEOUS INJECTION OF SMEGMA

A suspension containing 2.5 gm. of smegma in 25 cc. of lard filtered at 38° C. was injected (July 21, 1938) subcutaneously in the left axilla of 12 mice. The animals were about 3 months old and equally divided as to sex. About 0.35 cc. was administered, at monthly intervals at first and later at intervals of 6 to 8 weeks, until the poor physical condition of the mice precluded further injections. Abscess formation and ulceration occurred at the sites of injection. These required 3 to 4 weeks to heal and occurred less frequently following the later injections. The mice in this group each received from 7 to 9 doses. The first mouse died 9 months, and the last one 15 months, after the start of the experiment.

At post-mortem examination no tumors were found at the site of injection except one mammary adenocarcinoma, in a female mouse, which may have been spontaneous.

INTRAVAGINAL APPLICATION

Trauma control experiment

In 20 mice the vagina was dilated with a small forceps and a metal plunger was

inserted until it reached the cervix. The mice survived for 15 to 17 months from the start of the experiment during which this procedure was repeated 130 to 140 times. At post-mortem examination no gross evidence of induced tumors was found.

Smegma experiment

About 5 mg. of smegma was introduced (July 22, 1938) into the distended vagina of 20 other mice; with a plunger the smegma was gently forced upwards until it reached the cervix. This was carried out 2 or 3 times weekly for the first 12 months and less often thereafter because of the poor physical condition of the mice. The smegma did not remain in contact with the cervix, although it was retained in the vaginal canal. During the last quarter of the first year 5 mice died, after receiving 72 to 115 applications. The remaining mice died between the fourteenth and sixteenth months after receiving 130 to 140 applications. Post-mortem examination of these mice revealed no gross evidence of tumor of the genital tract.

Benzpyrene control experiment

A 5-percent solution of 3, 4-benzpyrene in cholesterol was prepared; when cold it was broken up into small fragments. According to Shear and Ilfeld (*1*) this concentration of benzpyrene in cholesterol was sufficient to produce tumors in the subcutaneous tissue of the mouse.[2] About 5 mg. of this preparation was introduced (July 30, 1938), as in the preceding experiment, into the vaginal canal of 10 mice 2 or 3 times weekly until a tumor appeared; thereafter it was repeated about once a week until the poor physical condition of the animal or obstruction of the vagina by a large tumor precluded further application.

The first tumors appeared in 2 mice during the seventh month. At the end of 10 months vaginal tumors had been produced in 6 mice, and by the end of 14 months all 10 of the mice had developed tumors at the site of application. The growths were first observed on the vaginal wall and grew progressively even after cessation of treatment, until the mice died or were killed in from 1 to 4 months after the tumors were first observed. The results are summarized in table 1.

PATHOLOGICAL OBSERVATIONS

Of the 10 mice in the group which received benzpyrene intravaginally, all developed carcinoma of the vagina which first appeared as a papillary growth. The tumors varied in size from 5 to 25 mm. in diameter, the average being about 10 mm. They arose in different sites in the vaginal wall, but all of them infiltrated the perivaginal tissues (figs. 1 and 2). Some tumors extended to the clitoral gland (fig. 3) anteriorly and the rectum posteriorly. Two protruded through the vaginal orifice.

TABLE 1.—*Rate of production of vaginal tumors in strain A mice by intravaginal application of 5 percent 3, 4-benzpyrene in cholesterol*

Mouse No.	First appearance of tumors		Death of mice	
	Time	Applications	Time	Total applications
	Months	*Number*	*Months*	*Number*
1	7	67	11	100
2	7	68	9	88
3	8	77	11	107
4	8	78	11	107
5	9	88	13	122
6	10	99	12	110
7	11	103	15	126
8	11	107	14	126
9	12	108	14	123
10	14	125	15	125

[2] *Of the 10 strain D mice employed in this experiment, 6 died early; tumors were induced in 4 animals. Shear (unpublished experiment, 1938) obtained similar results with 5-percent benzpyrene-cholesterol pellets introduced subcutaneously in strain A mice.*

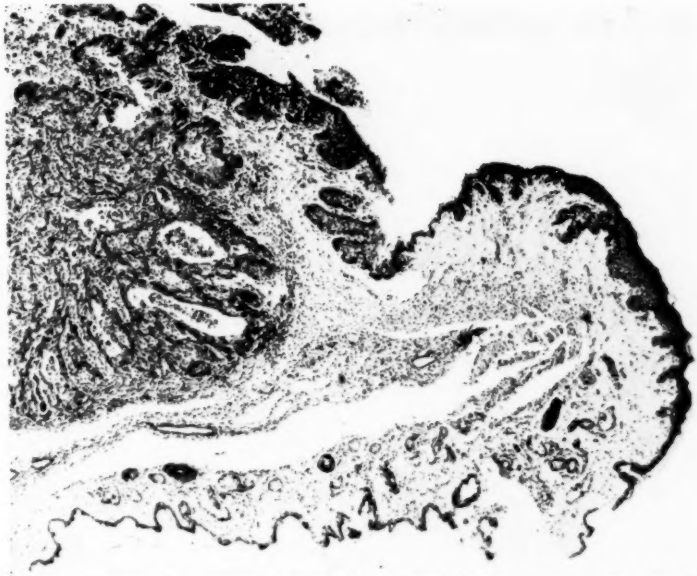

FIGURE 1.—Mucocutaneous junction of vagina showing nodule of carcinoma (5 percent benzpyrene in cholesterol). × 55.

In at least 1 case there was involvement of the mucocutaneous junction of the vagina. None of the tumors involved the cervix.

Microscopically, all the tumors were squamous-cell carcinomas composed of infiltrating cords and nests of basal, prickle,

FIGURE 2.—Junction of hyperplastic mucosa of vagina with carcinoma (5 percent benzpyrene in cholesterol). × 130.

FIGURE 3.—Carcinoma of vagina extending to clitoral gland (5 percent benzpyrene in cholesterol). × 30.

and flattened, squamous cells varying somewhat in size, shape, and staining qualities (fig. 4). Pearl formation and keratinization were pronounced in three. The remaining growths were cellular without much keratinization and contained areas in which the epithelial tumor cells were spindle-shaped. The tumor in one mouse was an adenoacanthoma. In three, the cells showed a pronounced papillary arrangement. Mitotic figures, numerous in all growths, averaged 4 to 5 per high-power field in one case. The stroma consisted of cellular or hyalinized connective tissue, scanty in amount, showing marked inflammatory reaction in four specimens, and marked vascularity in one. Ulceration and extensive necrosis were observed in 4 tumors.

TRANSPLANTATION

Two of the vaginal carcinomas were implanted in the subcutaneous tissue of other mice of the same strain. Although eight mice were used in each case, none of the transplants grew. Inasmuch as these tumors showed marked inflammatory changes microscopically, it is likely that infection may have accounted for the failure of

FIGURE 4.—Infiltration of perivaginal tissues by squamous-cell carcinoma (5 percent benzpyrene in cholesterol). × 130.

the transplants to establish themselves successfully.

DISCUSSION

The production of experimental carcinoma of the vaginal tract has been attempted by previous workers with an occasional positive result. Dobrovolskaia-Zavadskaia and Simsilevich (*2*) have reviewed the earlier literature [3] on the introduction of tar into the vagina of castrated and noncastrated rats; in their own experiments these authors introduced 1,2,5, 6-dibenzanthracene into the vagina of mice without obtaining any vaginal tumors, although they produced a tumor of the vagina in 1 mouse treated 44 times with tar. Klenitzky (*4*) recently applied coal tar and also "dibenzpyrene" to the vagina and uterine cervix of mice without producing carcinoma in 13.5 months. He obtained carcinoma of the uterine cervix in 2 of 170 mice treated with a combination of local application of coal tar and of repeated subcutaneous injections of folliculol. The effect of the coal tar is obscure inasmuch as he reported no data on the effect of repeated injection of the oestrus hormone alone in his mice. Perry and Ginzton (*5*) reported obtaining a vaginal carcinoma in a mouse which had received cutaneous applications of 1,2,5, 6-dibenzanthracene and parenteral administration of theelin; dibenzanthracene alone did not produce vaginal or cervical tumors. The role of long-continued administration of estrogen in the production of carcinoma of the uterine cervix of mice has been intensively studied by a group at Yale. Their most recent results are given in a paper by Allen and Gardner (*6*).

[3] *To this bibliography may be added the work of Fusco* (3), *who painted the vagina of rats with tar every 3 or 4 days; a squamous-cell carcinoma of the vagina developed in one rat in 10 months.*

Gardner [4] stated in a discussion that intravaginal instillation of a sesame-oil solution of benzpyrene or methylcholanthrene into mice gave rise to a considerable number of carcinomas of the skin of the external genitalia and perineum; the incidence of tumors of the vagina was "extremely low." Part of the injected oily material was usually seen to have escaped from the vagina. He stated: "It would seem that the vaginal epithelium had a strikingly different sensitivity than the skin."

Since spontaneous carcinoma of the vagina and of the uterine cervix in the mouse is rare (*7*), and since local application of chemical carcinogens to the vagina and cervix had not previously been reported as producing tumors in a high proportion of the animals treated, it was considered desirable to ascertain whether tumors in the vaginal tract could be produced in a high proportion of animals treated locally with a relatively weak carcinogenic preparation. A concentration of 5 percent benzpyrene in cholesterol was tried and gave satisfactory results.

The preliminary experiment with human smegma, however, did not give evidence of tumor-inducing potency. This may, of course, mean that smegma is not carcinogenic. But before accepting such a conclusion, we feel that further work with smegma is required.

Clinical data (*8–17*) on the incidence of carcinoma of the penis and of the uterine cervix have raised the question as to whether smegma has carcinogenic properties. According to Sorsby (*11*), penile carcinoma does not occur in men circumcized in infancy; it occurs only in uncircumcized individuals or in those circumcized after infancy, and is frequently

[4] J. Nat. Cancer Inst., 1: 502–504, 1941.

associated with phimosis; the incidence of carcinoma of the uterine cervix is much lower in women of religious or cultural groups in which the males are circumcized in early life than in women of other groups, in the same geographical areas, in which the males are not circumcized in early life.

According to the published data of a number of studies, the incidence of all uterine carcinomas is definitely lower in Jewish women than in non-Jewish. When the data for the cervix and uterine body are analyzed separately, the comparative infrequency of cervical carcinoma in Jewish women is more marked. Davidsohn's (*17*) figures for the incidence of cervical carcinoma, calculated as percentage of specimens examined, were 0.93 percent for Jewish and 6.4 percent for non-Jewish women. To date no adequate explanation has been offered for these observations.

In support of the hypothesis that smegma may be carcinogenic are the observations on penile carcinoma in the horse. According to Feldman's summary of the literature (*18*), the penis is, next to the eye, the most common site of carcinoma in the horse. Moreover, large amounts of smegma are found beneath the foreskin in the horse.[5]

The foregoing considerations seem to us to warrant further study of smegma, from both man and horse, from the point of view of possible tumor-producing potency. However, since it has not been feasible for us to prosecute this work further, this paper presents the results obtained in the preliminary experiments.

SUMMARY

The results of a preliminary investigation with 62 strain A mice are recorded. No tumors were induced by human smegma, administered subcutaneously or intravaginally. A preparation containing 5 percent of 3,4-benzpyrene in cholesterol, applied locally, produced vaginal tumors in all 10 of the treated mice.

The hypothesis that both cervical and penile carcinoma may, in part at least, be induced by smegma is briefly discussed.

[5] *Personal communication from Dr. Leo Weisz, Waltham, Mass.*

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The Early Stages of Carcinogenesis by 20-Methylcholanthrene in the Skin of the Mouse. I. Experimental Technique and Macroscopic Changes[1]

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The object of the observations recorded in this and the following paper (*1*), which represent contributions to a group investigation organized by Dr. E. V. Cowdry, was to obtain an insight into the manner by which methylcholanthrene produces its carcinogenic effect. A systematic study was made of the earliest stages of the changes induced in the mouse's skin by this, the most effective carcinogenic hydrocarbon.

Attempts directed toward such an end have been made repeatedly ever since the discovery of experimental carcinogenesis by tar more than 20 years ago. They have failed to give satisfactory results for a variety of reasons. The time necessary to induce cancer extends over a long period and the reaction of the skin to a given carcinogen shows extreme variations in different individuals, even when chemically pure carcinogens are applied to mice of a highly inbred strain under apparently similar conditions. With many of the carcinogens these individual differences are so marked that cancer can be induced in only a fraction of the treated mice. It has, therefore, become necessary to introduce a constitutional factor into the etiology of cancer which presents an additional problem for investigation. Lastly, if a carcinogen is applied to an extensive skin area, cancer does not arise uniformly throughout the whole tissue subjected to the carcinogen but in one or sometimes two or three sharply circumscribed centers, and it has been impossible to foretell where eventually this localized development of malignancy will occur.

Before such a systematic investigation could be carried out it was, therefore, necessary to establish a method of application of the carcinogen which would produce cancer of the skin (1) in a reasonably short time, (2) in a high percentage of the animals, and (3) in the center of the skin area subjected to the treatment. In a series of experiments the concentration of the methylcholanthrene solution, the frequency and the length of the period of its application, and the size of the skin area were varied. As a result of these preliminary experiments the technique described below was adopted.

EXPERIMENTAL TECHNIQUE

An inbred strain of mice, the Swiss strain, obtained from the Tumblebrook Farms, was used. The animals, males and females, were from 2–3 months old and weighed from 20–25 gm. They were kept on a diet of Rockland pellets. Methylcholanthrene was dissolved in benzene in a concentration of 0.6 percent. The solution was applied 3 times a week with a No. 4 brush, which was dipped into the solution. The excess fluid was removed by

[1] *Aided by a grant from an anonymous donor.*

shaking the handle of the brush against the mouth of the bottle, and the solution was applied to the nonepilated skin by a single brush stroke beginning at the nape of the neck and carried towards the rump along the midline for a distance of about 1.5 cm. The skin area directly affected by this procedure was about 0.5 cm. broad and 1.5 cm. long. By capillarity a part of the solvent containing some of the carcinogen spread from this area mainly to the sides and to the back, so that the total skin area exposed to contact with the carcinogen was about 1.0 to 1.5 cm. broad and 3 cm. long. In order to get an approximate idea of the amount of methylcholanthrene applied to the skin at each brush stroke, similar brush strokes were applied to a weighed piece of blotting paper, which is highly absorbent, and to a weighed piece of less absorbent typing paper. It was found that 0.1 mg. was delivered with each brush stroke when blotting paper was used, while with the less absorbent typing paper 0.07 mg. was delivered. Since 42 paintings were made during 14 weeks, it follows that under our experimental conditions the total amount of the carcinogen applied was about 4 mg. As will be seen later, the area of skin to which the carcinogen solution was applied directly by the brush was more profoundly affected than the surrounding area of skin to which the solution had been spread by capillarity, and with very few exceptions, the malignant tumors appeared in the first-mentioned area.

This technique was carried out in a group of 36 mice for a period of 14 weeks, when a few mice had developed malignant tumors and the application was then discontinued. The animals were examined carefully once a week from the beginning throughout the experimental period for skin changes visible to the naked eye. These will be described in more detail presently. After 24 weeks all of the surviving 28 mice had developed malignant tumors, and these malignant neoplasms appeared in the great majority of the mice, 18, within a short period—between the fourteenth and twentieth week.

It is possible to obtain an even better result so far as the shortening of the induction period of cancer is obtained. In another experimental series, with a 0.6 percent methylcholanthrene solution in benzene we induced malignant tumors in 100 percent of the survivors in a period of 18 weeks by enlarging the area of skin subjected to the carcinogen. But such an enlargement has obvious disadvantages in the microscopic study of the earliest skin changes. It might seem that for such a purpose a limitation of the application to the smallest possible skin area would be the ideal. We found, however, that a reduction in the skin area exposed to the carcinogen has the disadvantage of extending the latent period preceding the development of malignancy over a much longer period and of diminishing the percentage of animals in which it occurs. Another factor which was found to prolong the latent period and to reduce the percentage of malignant growths was a reduction in the concentration of the methylcholanthrene solution.

The technique described also fulfills the third requirement mentioned above, the appearance of the malignant tumors within the center of the skin area subjected to the carcinogen. This result mainly depends on the care with which the brush is applied in a single stroke to the midline of the back. If this is observed, benign tumors develop along the midline of the back or very close to it, and so do eventually malignant tumors. The animals were examined carefully once a week. Any changes were noted in the protocols, and the growth was charted of the tumors that

appeared, special attention being given to the presence of features indicating the development of malignancy.

The first effect visible to the naked eye was epilation of the skin area subjected to the carcinogen. In more than half of the animals epilation was present within 3 days after the first application. The males of the Swiss strain are more resistant to the epilation by methylcholanthrene than the females. Under our experimental conditions this epilation persisted throughout the period of application of the carcinogen. In most of our animals subjected to repeated paintings the skin remained epilated even after the application of methylcholanthrene had ceased. In some animals there was at the beginning of the course of painting a partial but very incomplete regrowth of hair.

From the beginning of the experiment there was a dilatation of the subcutaneous blood vessels visible to the naked eye when the skin was excised. It became increasingly intense with the changes in the skin leading to the development of a malignant neoplasm. This change, to which Kreyberg (*2*) first drew attention and which has recently been emphasized by Orr (*3*), is due mainly to the carcinogen. But it should be noted that benzene alone is able to induce a considerable degree of vasodilation, so that the vasodilation induced by a benzene solution of a carcinogen is a mixed effect. The specific vasodilating effect due to carcinogens can be demonstrated by dissolving them in acetone. Acetone by itself does not produce a dilatation of the subcutaneous blood vessels, while this change is induced by an acetone solution of methylcholanthrene. Shallow extensive ulceration, not due to an underlying malignant tumor, was observed by us sometimes in the skin area subjected to the painting with methylcholanthrene and sometimes also outside that area. Such ulcerations may persist for some time, but they may also heal over with surprising rapidity. Orr has also reported the occurrence of these conditions. They do not appear to have any direct connection with the subsequent development of carcinomas.

The most important macroscopic skin changes, from the point of view of carcinogenesis, are the appearances of swellings, usually described in the literature on experimental carcinogenesis as "tumors."

The term "tumor" is being used very loosely in the recent literature on experimental carcinogenesis and not always in the sense given to it by pathologists. Reference to this point will be made later. Here we wish to state that on the basis of our systematic observations on the macroscopic changes in the skin reported herein and also on the microscopic changes, which will be given in a subsequent paper, it is necessary to distinguish between different types of swellings appearing in the mouse's skin after the application of methylcholanthrene because they differ biologically and morphologically.

In the pathology of cancer the term "tumor" has a definite meaning. Ewing (*4*) begins the second chapter of his book with the definition: "A tumor is an autonomous new growth of tissue." The property of autonomous growth manifests itself in experimental carcinogenesis by the continued growth of the tumor, when the agent which has given rise to it ceases to act. When to the property of autonomous growth, which, when present alone, characterizes a benign tumor, is added the property of infiltrative invasive growth, the tumor is a malignant one. Morphologically, true tumors are characterized by being composed of one particular type of cells, epithelial, mesothelial, or endothelial, as the case may be, and by a supporting

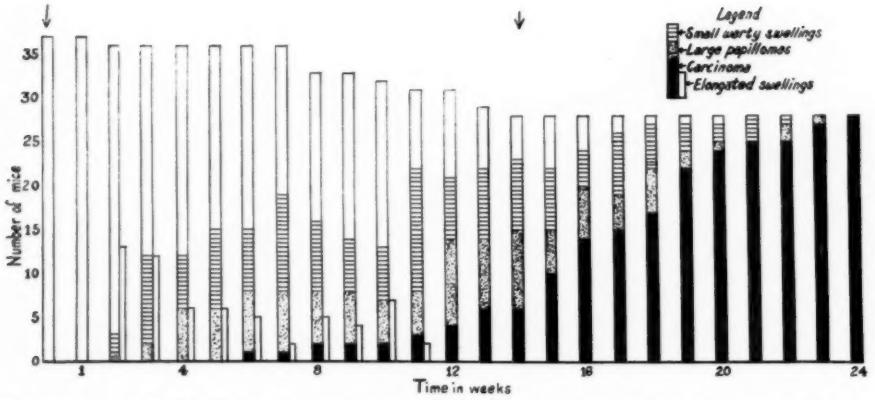

FIGURE 1.—Results of painting a moderately large sized area of the back of Swiss mice with 0.6 percent methylcholanthrene in benzene thrice weekly.

stroma, and they are diagnosed as true tumors by virtue of their morphologic criteria.

The course of macroscopically visible changes observed in the skin subjected to the application of methylcholanthrene under the experimental conditions detailed in this paper is represented graphically in figure 1. In this figure we have distinguished as far as possible between four types of swellings: (1) Elongated swellings; (2) warty swellings; (3) benign tumors; and (4) malignant tumors. Now a benzene solution of methylcholanthrene, when applied to a large area of skin, produces, in addition to true tumors, other swellings which do not possess either the biologic or morphologic characters of true tumors. These swellings differ biologically because they do not possess the property of autonomous growth. They regress when the application of the carcinogen is discontinued and even during the continued application of the carcinogen. They differ morphologically because the elevation above the level of the normal skin which gives the impression of a tumorlike formation is due to an edematous swelling of the dermis pushing the epithelium, which shows only a slight degree of hyperplasia, away from the panniculus carnosus.

The swellings which are not true tumors appear as two different types. One type, which under our experimental conditions develops as a very early reaction to the carcinogen, is extensive. It may present itself as an elongated ridgelike elevation with a narrow plateau elongated for a considerable length along the midline of the back in the direction of the brush stroke and covering a large part of the skin area to which the brush has been applied. The ridge may be continuous, or it may be divided into several sections by narrow transverse depressions, producing thus in the midline a row of swellings elongated along the long axis of the animal. Another modification of these ridgelike elevations is the appearance of two narrow ridges running parallel and close to each other on either side of the midline, being separated from each other by a narrow, deep depression.

This type of swelling appeared very early and in considerable numbers. Thus in a series of 36 mice in which a 0.6-percent

solution of methylcholanthrene in benzene was applied 3 times weekly to a large skin area, 13 mice developed these elongated swellings at the end of the second week. In another experiment on 40 mice similarly treated with a 0.3-percent solution of methylcholanthrene in benzene, 19 mice developed elongated swellings at the end of the second week. Even a single application of a 0.6 percent methylcholanthrene solution to a large skin area was sufficient to induce these swellings in about the same percentage. The appearance of these elongated swellings was observed only when the methylcholanthrene was applied to a large skin area. They did not appear when the carcinogen was applied to a small skin area. This indicates that the swelling of the dermis which is responsible for this reaction is the result of a mass action when a large area of skin is subjected to the effect of a relatively high concentration of the carcinogen.

All these elongated swellings undergo a process of regression, owing to an unevenly distributed shrinkage. This either leads to their complete disappearance, or an isolated, small, semiglobular, warty swelling remains. This regression takes place whether the application of the carcinogen is continued or not. Such semiglobular warty swellings represent the second type of nontumorous swellings. They may appear directly without the previous formation of the more extensive elongated swelling, and they develop at a somewhat later stage, though still early in the experiment. The epithelium shows a slightly more advanced stage of hyperplasia, but their tumorlike elevation above the level of the normal skin is also mainly due to a swelling of the dermis and not to a massive hyperplasia of epithelial cells growing autonomously. Although it is sometimes difficult to identify this type from its macroscopic appearance and to distinguish it from an early benign tumor, it can be recognized, if weekly chartings are made, by its regression when the application of the carcinogen is discontinued. These swellings may regress even under continued application of the carcinogen, or a benign tumor may develop in them when the epithelium has undergone a sufficient degree of hyperplasia in response to the continued treatment with the carcinogen. A true tumor, either benign or malignant, may originate also from the hyperplastic epithelium of any other part of the treated skin without any previous formation of the swellings just described. The malignant skin tumors appear anywhere along the midline in isolated, sharply circumscribed centers. They develop frequently from the base of a preexisting papilloma, but a carcinoma may also develop in a small skin area in which there is no papilloma. Two and even more carcinomas may be present at the same time in separate, sometimes widely separate, parts of the skin. The significance of these features of carcinogenesis in a large skin area subjected to the action of a carcinogen has been previously presented by one of us (*5*).

The diagnosis of malignancy was always made by histologic examination. As soon as we felt sure from inspection that a tumor had become malignant, the animal was killed, the whole skin area was excised, and transverse and axial sections through the breadth and length of the painted skin area were made. In several animals multiple carcinomas could be seen in different stages of development; in three animals a carcinoma was associated with a spindle-cell tumor. Two of these spindle-cell tumors were probably sarcomas, since they had appeared separately from the carcinoma and underneath a part of the skin where the epithelial hyperplasia was slight.

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It is interesting to note that these two tumors developed, not in the midline but slightly to one side of it. The third spindle-cell tumor was in close contact with a carcinoma of undoubtedly epithelial origin developing in the midline and was possibly a spindle-cell carcinoma.

The three other groups of swellings were diagnosed from their macroscopic appearance. The elongated transient swellings were unmistakable, but it was more difficult to distinguish a wartlike transient swelling from a true benign tumor. This was especially the case in the later stages of the experiment when a benign tumor might arise from a wartlike swelling. The number of wartlike swellings may, therefore, diminish for two different reasons: (1) They may undergo regression; or (2) they may become transformed into a benign tumor. As a result the number of these swellings fluctuates widely and irregularly as seen in figure 1. The number of benign skin tumors may be said to diminish during the treatment with methylcholanthrene for only one reason, namely, that a malignant tumor arises in a part of their hyperplastic epithelium, although such benign tumors occasionally regress spontaneously after the treatment with carcinogen has ceased. In the graph this is reflected in an increase in the number of these tumors during the first half of the experiment and a decrease thereafter. The general aspects of the results will be commented on in the following section.

After a suitable technique was selected it seemed desirable to investigate at the same time the early skin changes following a single application of methylcholanthrene. Another experimental series of Swiss mice received one application only of a 0.6-percent solution of methylcholanthrene in benzene, the same technique being used. A single application was sufficient to produce epilation in many animals in the first week. It also induced, in the second week, the elongated type of swelling described above as appearing after the repeated application of methylcholanthrene. Subsequently these transient swellings underwent the same process of shrinkage, which led in most animals to a complete disappearance of these swellings, but which proceeded in some individuals to the formation of a benign tumor.

Since all the animals of this group were killed for the histologic study of the early changes, which, as described in the following paper, revealed an unexpectedly active response to a single application of methylcholanthrene suggesting the possibility of a later development of malignancy, a second group of 20 mice were subjected to a single application of methylcholanthrene to the skin. The animals of this group were allowed to live. The experiment is not yet completed, but so far 3 of these animals have developed a carcinoma, 1 in the fourth, 1 in the fifth, and the third mouse in the seventh month, while in a fourth animal a sarcoma developed after an interval of 3 months. Previously Mider and Morton (*6*) recorded the development of papillomas and also of a few carcinomas after a single application of methylcholanthrene to a very large area of skin of mice belonging to one strain, the C57 Brown. The carcinogen was applied in a 0.5-percent solution of benzene. In 156 mice, 7 carcinomas developed between the fourth and the eighth month. These results obtained in 2 different strains of mice in 2 different laboratories demonstrate that a single application to the skin of mice has a more prolonged effect than has been suspected, provided that a potent carcinogen is used and that a large area of skin is exposed to it.

EFFECT OF VARIATION IN TECHNIQUE IN THE CARCINOGENIC RESPONSE

In our search for a technique giving a near optimal carcinogenic response, a number of different techniques were tested. Two of them were carried out on groups of mice sufficiently large to make possible a comparison with our standard technique. In one the concentration of the methylcholanthrene was lowered to 0.3 percent and only two applications a week were made, while the area of skin exposed to the carcinogen was the same as in our standard technique. In another group, the concentration of the carcinogen in benzene and the number of applications per week remained the same as in our standard technique, but the area of skin to to which the carcinogen was applied was reduced to one-fourth of that exposed under the conditions of our standard technique. In this way the total amounts of carcinogen applied to the skin were necessarily varied in the three experimental series, but the amounts applied per unit area of the skin did not vary correspondingly. Per unit area the amounts of carcinogen were about the same in the two series in which a 0.6-percent solution was applied 3 times weekly, while they were much smaller in the third series in which a 0.3-percent solution was applied twice weekly. Figure 2 gives the results showing the number of animals in which carcinoma developed, expressed in percent of the effective mice. It is surprising to find that the size of the exposed skin area determines the carcinogenic response to a greater extent than the concentration of the carcinogen, the frequency of its application, or the total amount of carcinogen administered.

DISCUSSION

In our experiment the development of malignancy in the epithelium of the mouse's skin in response to methylcholanthrene followed a curve very similar to that recorded by Andervont and Shimkin (*7*) for the development of malignancy in the connective-tissue cells of the mouse's skin in response to the same carcinogen by a different technique, which they recommend for the assay of the carcinogenic potency of chemical compounds. In Andervont and Shimkin's technique, the carcinogen was introduced subcutaneously and was therefore acting continuously and throughout the whole period of the experiment. In our technique, the application of the carcinogen was intermittent and being restricted to a limited period ceased before some of the animals had

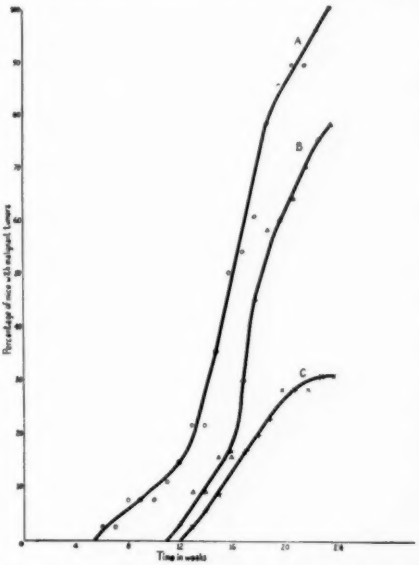

FIGURE 2.—Percentages of Swiss mice developing malignant tumors following the application of methylcholanthrene in benzene for 14 weeks. *A*, 28 mice painted with 0.6 percent methylcholanthrene thrice weekly over a large-sized area of the back; *B*, 33 mice painted with 0.3 percent methylcholanthrene twice weekly on a large area of the back; *C*, 36 mice painted with 0.6 percent methylcholanthrene thrice weekly on a small area of the back.

developed malignant neoplasms. This difference in technique and the greater liability of the connective-tissue cells of the mouse to malignant transformation, as a result of which substances which are not carcinogenic for the epithelium of the skin are able to induce sarcomas when implanted subcutaneously, account for the greater rapidity of the development of sarcomas recorded by Andervont and Shimkin. While the average latent period for sarcomas is shorter than for carcinomas, it is interesting to note that the first malignant tumors appeared at about the same time in both investigations, namely, in 6 weeks. It is probable, therefore, that this interval approaches the minimum latent period for the development of malignancy in mice.

There are many different ways of applying a chemical carcinogenic agent: by means of a brush, of a pipette, of a pointed stick or glass rod, or by pressing a surface moistened with the solution against the skin. The size of the brush may vary, and the carcinogen may be applied either by a single brush stroke along a strip of skin or by several strokes over a broader area; the pipette may be a micropipette or an ordinary pipette; the size of the moistened surface may be large or small. Such variations in the technique affect not only the size of the skin area subjected to the carcinogen but also the absolute amount of the carcinogen administered at each application. We have not carried out a systematic experimental comparison between all the different techniques mentioned, but in our search for a near optimum carcinogenic response, we have used a number of different techniques and have found that even slight variations in technique are sufficient to alter the carcinogenic response. The importance of such differences in technique does not appear to have been recognized sufficiently, since, apart from giving the concentration of the carcinogenic solution and the frequency of weekly paintings, details of the technique are frequently missing, even in the record of experiments in which quantitative comparisons are made.

Our observations conform to the results obtained by Dunning, Curtis, Wood, and Eisen (*8*, *9*) in their extensive investigations on the development of sarcomas in response to inocula of definite but varying sizes containing definite but varying amounts of a carcinogen. In the skin as in the connective tissue, the factors favoring carcinogenesis are not only an increase in the concentration of the carcinogen, but also with one and the same concentration the area of tissue exposed to the carcinogen, together with an increase in the amount of the carcinogen delivered at each application.

An even more serious error is the recording of so-called tumors as a measure of the carcinogenic effect of an agent. As has been pointed out in this paper, it is often difficult to distinguish between a nontumorous swelling and a true benign tumor. Furthermore, the production of a true benign tumor is not necessarily a measure of the carcinogenic potency of an agent, for it is possible to induce benign tumors which persist as such and do not become malignant. If we have dealt at some length on the necessity of distinguishing between true benign tumors and nontumorous swellings in the experimental production of skin cancer, it is because at the beginning of this investigation the rapid appearance of massive swellings led us to believe that we had induced rapidly a precancerous condition, and the subsequent disappearance of these swellings seemed perplexing. The histologic examination of these early changes, which will be dealt with in the subsequent paper, afforded an explanation.

SUMMARY

The numerous papers on experimental carcinogenesis in the skin contain little, if any, reference to experimental details of the technique of application of the carcinogen, even when a quantitative comparison is made for the purpose of identifying factors either favoring or inhibiting the development of malignancy. Experiments were carried out in a search for a technique which would give a near optimum carcinogenic response regularly and in a predetermined skin area. It was found that even slight variations in the technique affected materially the carcinogenic response.

For the initiation of the malignant process in the skin of mice by repeated applications of methylcholanthrene, a technique is described by which cancer can be induced in 100 percent of the mice, with a low mortality and with a latent period having a minimum of 6 and a maximum of 24 weeks. Within this period of 18 weeks the initiation of malignancy was not evenly distributed but was concentrated within the short period of 8 weeks, more than two-thirds of the malignant tumors developing between the twelfth and the twentieth week. All the carcinomas developed within the circumscribed area of the skin directly subjected to the action of the carcinogen. The size of the skin area exposed to the carcinogen is a factor determining the carcinogenic response.

A single application of methylcholanthrene to a large skin area produced papillomas and even carcinomas. This result, which is in agreement with observations of Mider and Morton in a different strain of mice, demonstrates that the effect of a single external application of methylcholanthrene to the skin is much more persistent than hitherto suspected.

Attention is drawn to the pathologic nature of tumorlike swellings which appeared in the skin under our experimental conditions. Many of these swellings could be recognized as not being true tumors in the sense in which the term "tumor" is used in cancerology, because they regressed even while the treatment was being continued. They were due to an edematous swelling of the dermis. Some were very massive, differing in this respect also from true benign tumors, while others less massive closely resembled benign tumors. The nature of these swellings could not be established without a histologic examination and such examination was not as a rule made until a malignant tumor had appeared. This is one indication that in experimental carcinogenesis the appearance of so-called tumors or papillomas is not a reliable measure of the carcinogenic response, although it is frequently used as such.

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The Early Stages of Carcinogenesis by 20-Methylcholanthrene in the Skin of the Mouse. II. Microscopic Tissue Changes[1]

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INTRODUCTION

The technical difficulties which have so far prevented a systematic study of the mode of action of the chemical carcinogens have been discussed at the beginning of the preceding paper (*1*). They have been overcome to a certain extent by the technique of application, described in that paper, to which skin responds with a near optimal carcinogenic effect, as measured by the conventional criteria. But even with that technique individual animals still show striking differences in the reaction of the skin to the carcinogen. Thus the earliest carcinomas develop between the sixth and tenth week at a time when in many animals the skin shows no macroscopic or microscopic evidence of early changes which could be interpreted as precursors of a subsequcnt development of a carcinoma. Even with the standardized technique, the response of the skin is still so erratic and capricious that it is not possible to obtain a series showing gradually progressive changes by the obvious method of applying the carcinogen to the skin of a large number of animals and examining the skin at regular short intervals. This difficulty was diminished when it was found—quite unexpectedly—that the skin reacts more uniformly to a single application of the carcinogen, and—what was even more surprising—that the changes induced in the skin by a single application can be progressive over a long period. We have since found that skin cancer can be induced more uniformly and effectively in relation to dose by a small number of applications given at long intervals than by the conventional method of numerous applications at short intervals (Cramer and Stowell (*2*)). As stated previously (*1*), we confirmed Mider and Morton's observation (*3*) that in some individual mice a single application is sufficient to induce cancer.

In the present investigation, a study was made of the early changes taking place in the skin during the first 4 weeks after a single application of methylcholanthrene to the skin. The information thus obtained was then used as a guide to the interpretation of the early changes resulting from the application of the carcinogen repeated every second day as described in our standard technique over a period of 8 weeks, when carcinomas began to appear.

EXPERIMENTAL MATERIAL

In a series of 50 mice of the Swiss strain a 0.6 percent solution of methylcholanthrene in benzene was applied. A second application was given 3 days later, when in most of the mice the painted skin area had become epilated, and after that the

[1] *Aided by a grant from an anonymous donor.*

carcinogen was applied every second day during 12 weeks. In a second series of 25 mice only 1 application of methylcholanthrene was given, so that in this latter series the effects of a single application of the carcinogen, which only exceptionally induces cancer, could be studied as a comparison with the effects of repeated applications of the carcinogen, which always lead to cancer. In the series receiving repeated applications of the carcinogen, 2 mice were removed after each application of methylcholanthrene during the first month of the experiment and killed with chloroform 3 days later. The condition of the skin was therefore examined always 3 days after each application of methylcholanthrene, as shown in the following tabulation in which the day of the first application is zero. In the second and third month of this experiment only 1 animal was killed after each application. At the end of the experiment the 4 surviving mice had developed carcinomas.

Days after first application

Painted—0 5 5 7 9 11 13 . . . .
and so on
Killed—3 6 8 10 12 14 16 . . . .

During the first month, 2 or 3 mice belonging to the experimental series receiving only a single application of the carcinogen were killed on the same days for microscopic examination of the skin. The series receiving only 1 application of the carcinogen was completed on the twenty-eighth day after the single application. In order to bring out the potential mitotic activity of the cells in the different stages, the animals received 0.025 mg. of colchicine 4½ hours before death. The interval of 3 days between the last painting and the death of the animal was chosen because, as will be shown presently, a specific reaction is produced in the epithelium 3 days after a single application of the carcinogen.

For the histologic study of the skin changes, the following procedure was adopted: An area of skin slightly exceeding that exposed directly or indirectly to methylcholanthrene was excised. In the series receiving repeated applications, the affected area was clearly delimited by its epilation. In the series examined after a single application, this was also evident in the earlier stages, but later the hair had partly regrown, and epilation, if present, was restricted to a narrow area along the middle line of the back. The skin was stretched out gently on mica plates and fixed in Bouin's fixative for 24 hours.

In addition, the earliest changes occurring during the first week were re-examined in a series in which 3 mice were killed every day during the first week. Our histologic observations are thus based on the study of 94 different specimens of skin which had been subjected to an increasing number of applications of methylcholanthrene.

MICROSCOPIC CHANGES

Technique

The significance of the sequence of changes in the skin induced by methylcholanthrene will be appreciated most readily by describing first the changes following upon a single application of the carcinogen. In order to appreciate the significance of the account which follows and the terms used therein, it is necessary to give a detailed description of the technique used. A relatively long single brush stroke is carried along the back of the animal in its long axis with the result that the bulk of the carcinogen solution is applied to the skin coming directly into contact with the brush, while some of the solution spreads by capillarity to either side. The first application gives rise to two different reactions in the epidermis which are described in detail in the follow-

ing section. In the skin area subjected to direct contact with the brush, there is a toxic effect involving the destruction of living tissues. This area will be referred to as the central area. In the surrounding area there is a differentiation of the epithelium with an increase in the layers of cells building up the epidermis. This area will be designated as the peripheral area.

The skin was examined in sections carried along two different directions. One, the transverse section, represents the skin at right angles to the brush stroke; the other, the axial section, is cut in a direction parallel to the brush stroke. This device makes it possible to compare sections of the skin of different individuals having the same relationship to the brush stroke. In the transverse sections the central area was necessarily in the middle of the section flanked on either side by two peripheral areas, while outside those was normal skin which had not been subjected to the action of the carcinogen. The transverse sections give, therefore, a representative picture of the skin changes in all the different phases taking place in an individual animal at one moment. Most of the descriptions refer, therefore, to such transverse sections, unless stated otherwise. The axial sections may go through either the central area or through one of the two peripheral areas, with the result that axial sections from the skin of an individual animal may present very different pictures according to whether they pass through the central area or one of the two peripheral areas. The axial sections present, therefore, a more homogeneous picture along their lengths; but the picture from the same skin specimen will vary widely according to the area through which it happens to pass, especially in the early stages of the experiment. If the section passes through the central area, it may include a destructive effect. If, on the other hand, it passes through the peripheral area, the destructive effect will be absent, and the skin will show a differentiated hyperplastic epithelium.

It is essential, therefore, to fix the direction in which the carcinogen is applied to the skin so as to be able to orientate the direction along which the skin is sectioned. Unless this is done, the pictures of the skin changes in the early stages are very confusing. Since in other publications dealing with the early stages of carcinogenesis no account seems to have been taken of these factors, it seems desirable to emphasize the importance of this technical point. The advantage of axial sections as compared with transverse ones is that the hair follicles are cut longitudinally instead of transversely. The axial sections present a better picture of the changes taking place in the hair follicles. For the study of the later stages of experimental carcinogenesis, they have the further advantage of demonstrating clearly the fact that an area of skin exposed to the action of a carcinogen over a prolonged period does not react equally to the carcinogen but shows considerable variations in the degree of response. The final carcinogenic response is restricted to one, or sometimes several, sharply localized centers. Elsewhere in the skin there may be at the same time only a moderate degree of hyperplasia while in another localized center the epithelial hyperplasias may have progressed to a precancerous condition. This point is illustrated in figure 2 and will be discussed later.

Effects of a Single Application

The normal skin of the mouse presents two layers of small undifferentiated epithelial cells. Mitotic division in these epithelial cells of the epidermis is infrequent even when colchicine has been given previously,

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but it is more frequent in the bulbs of the hair follicles. The immediate and most obvious effect of a single application of a 0.6-percent solution of methylcholanthrene in benzene is a transformation of the epidermis. The epithelial cells enlarge and undergo a differentiation into basal cells, spinous cells, and cells of the stratum granulosum and stratum corneum. The mouse epidermis assumes, therefore, the appearance of an epidermis consisting of several layers of cells undergoing a process of differentiation, as seen in man and most other mammals. This reaction is, however, not specific for carcinogens. As pointed out later (p. 392), it can be induced also by other noncarcinogenic agents. The differentiated epithelium produced by the application of the carcinogen shows minor differences from a normal differentiated epithelium in irregularities of the layers, irregularities and variations in the sizes of the cells and of the nuclei, and unequal staining of the central area. This differentiated epithelium persists for the first 2 days after a single application of the carcinogen. There is also the beginning of a hyperkeratosis. On the third or fourth day the cells of the central area degenerate and break down, leaving a single layer of thin elongated epithelial cells protected by a relatively massive layer of keratin (pl. 1).

Since the normal epidermis of the mouse consists of only two layers of cells, the change to an epidermis consisting of five to six layers might be described as a hyperplasia, but for two considerations. It is a generally accepted biologic axiom, based mainly on embryologic findings but observed also in cells cultured *in vitro*, that there is an antagonism between cell division and cell differentiation. The phenomenon observed occurring immediately ou the application of a carcinogen or of benzene, if attributed to cell division, would represent a reversal of this axiom. Actually, however, and this is the second consideration, the increase in the number of cells is not accounted for by a correspondingly increased mitotic activity of the epidermal epithelium. Whatever the explanation of this paradoxic phenomenon may be, a distinction should be made between the early nonspecific reaction, namely, an increase in the number of epithelial cells showing the normal arrangement of the epidermal epithelium in the different layers, which is essentially a process of differentiation and will be described as such, and the specific response to a carcinogen, namely, the excessive proliferation of epithelial cells occurring at a later stage showing disturbances of the normal arrangement in layers, which is a true hyperplasia.

Since the degenerative process affects only the central area, while in the peripheral areas the differentiated epithelium is maintained, the transverse sections at this stage show a central area of degenerated epithelium flanked on either side by areas of differentiated epithelium. The skin external to these peripheral areas where it has not been subjected to the action of the carcinogen has maintained the undifferentiated two-layered epithelium characteristic for the normal skin of the mouse. As just stated, the formation of the differentiated epithelium with five or six layers of cells in the peripheral areas is not accompanied by a correspondingly marked increase in mitotic activity, which, if it were present, would have been emphasized by the use of colchicine. The differentiated epithelial cells show only slight irregularities in their arrangement; they vary in size, and some of them show slight nuclear abnormalities. The hair follicles in the central area and in the peripheral areas undergo changes corresponding to those observed in the epidermis. In the central area the hair follicles undergo a process of

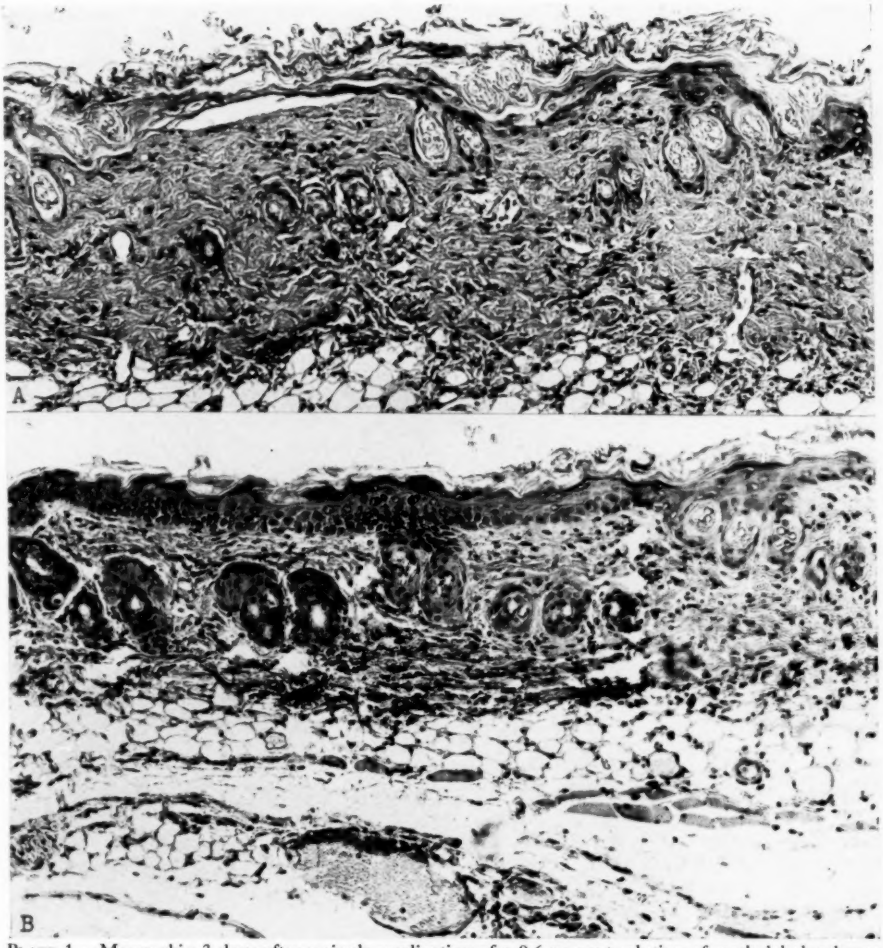

PLATE 1.—Mouse skin 3 days after a single application of a 0.6-percent solution of methylcholanthrene in benzene. *A*, from center of painted area, showing partial destruction of epithelium and hair follicles; *B*, from margin of painted area showing beginning of differentiation of epithelium. Transverse sections. × 140.

extensive degeneration which in some animals leads to a complete and permanent disappearance of many follicles. As a result, the central area remains epilated for many weeks after a single application of the carcinogen. In the peripheral areas the hair follicles undergo a partial and incomplete change which affects their functional activity without leading to a disappearance of the epithelial cells constituting the follicles. The hair falls out, but the cells persist, losing temporarily the power to form hair and forming elongated epithelial projections stretching like papillae down into the dermis from the differentiated epidermal epithelium. These differ-

ences in the hair follicles are seen more clearly in the axial sections than in the transverse sections (pls. 2 and 3, *A*).

At the end of the first week, regeneration of the epithelium in the central area begins. In this regeneration the epithelial cells of the altered hair follicles in the peripheral areas take a prominent part, as demonstrated by the frequent mitoses seen in these papillary projections. Some mitoses occur also in isolated cells of the epidermal epithelium. As a result, epithelilal cells, assuming at first elongated forms, appear in increasing numbers repairing the epithelial defect until the defect is covered by a many-layered epithelium composed of differentiated cells.

These changes are accompanied by a

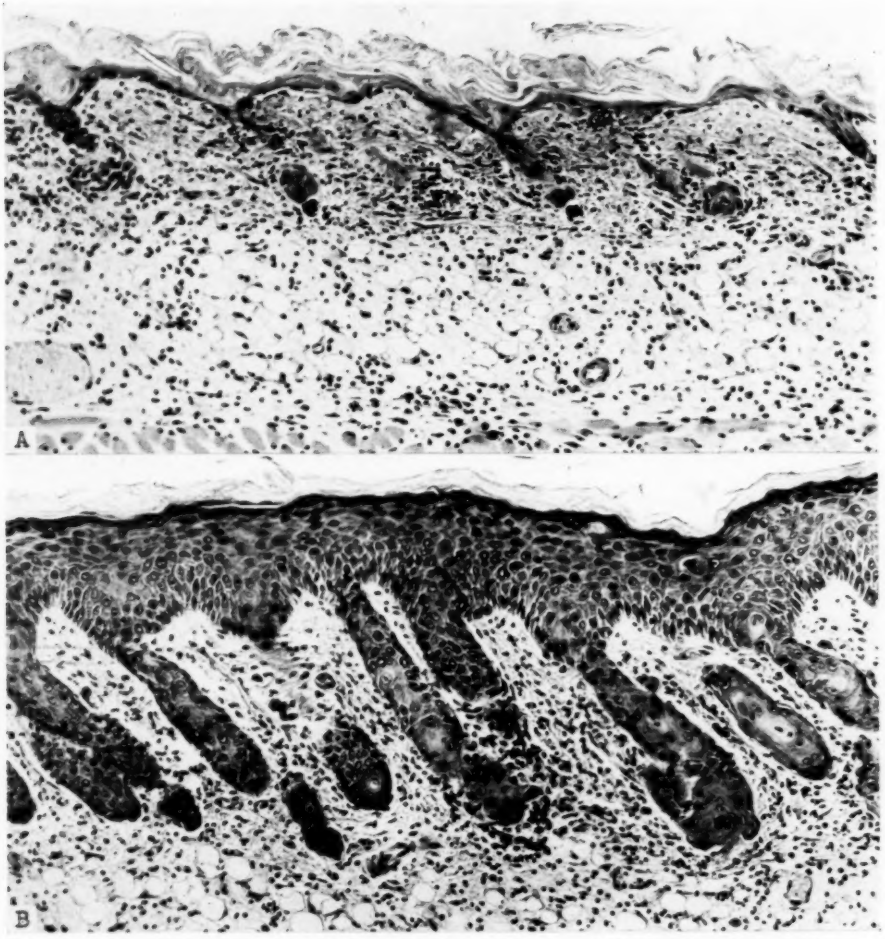

PLATE 2.—Mouse skin 6 days after a single application of a 0.6-percent solution of methylcholanthrene in benzene. *A*, from center of painted area showing thin layer of elongated epidermal cells and hair follicles undergoing resorption; *B*, from margin of painted area showing differentiated epidermis and regenerating hair follicles with mitoses. Axial sections. × 140.

PLATE 3.—Mouse skins after single application of a 0.6-percent solution of methylcholanthrene in benzene. After, *A*, 6 days; *B*, 14 days; *C*, 22 days; *D*, 26 days; and *E*, 31 days. Transverse sections. × 13.

hyperkeratosis on the surface, while the dermis is invaded by a variety of wandering cells including macrophages. The macrophages may take an active part in the destruction of the severely damaged hair follicles in the central area, while in the peripheral areas they seem to be responsible for cutting off some of the papillalike epithelial projections from the epidermis so that isolated masses of epithelial cells derived from the hair follicles are left lying in the dermis. There is also in the dermis a marked vasodilation of the smaller blood vessels and of the lymphatics.

In the second week, the regeneration of the epithelium in the central area is completed. This is accompanied by a greatly increased mitotic activity in the bulbs of the hair follicles in the peripheral areas flanking the central area. Many of these hair follicles, which have increased greatly in length and in thickness, resume their normal functional activity of hair formation. The regeneration of the epidermal epithelium in the central area may not go beyond the formation of a differentiated epithelium consisting of a fairly regular arrangement of five or six layers of cells, but in many of the animals the regeneration is irregular and excessive. In the following period, the mice which have received a single application of the carcinogen fall, therefore, into three main groups. In one the reaction remains stationary and consists mainly in the formation of a differentiated epidermal epithelium with a complete or partial atrophy of the hair follicles in the central area which remains epilated. At the other extreme are the animals with progressive changes. Between these two main groups are the mice with intermediate changes.

The changes in this group with progressive changes are as follows: Cell division becomes more frequent and is seen mainly in the epithelial stumps derived from the transformed hair follicles where dividing cells are in groups close together. The epidermal epithelium increases in thickness with an abnormal distribution of the different types of cells, and in the sizes of the cells belonging to one particular type. Some hair follicles form cystic spaces distended with keratin and lined by a single or a double layer of epithelium, others undergo a change to broad masses of epithelial cells either attached to the epidermal epithelium or lying free in the dermis. The keratin-filled cysts (pl. 3, *B*) may open to the surface, or they may lie in the dermis completely enclosed by the lining epithelium. The change has now reached a stage of an epithelial hyperplasia. This hyperplasia progresses in the third and fourth week leading to irregular epithelial formations, showing bizarre distentions of the solid epithelial masses lying in the dermis and a thickening of the epithelial lining of the keratinized cysts, both structures derived from the hair follicles (pl. 3, *D* and *E*). In this group the hyperkeratosis increases progressively and roughly in proportion to the degree of the epithelial hyperplasia. The specimen shown in plate 3, *C*, illustrates the structure of the small wartlike swellings which, as described previously (*1*), appear at an early stage of the treatment with methylcholanthrene. In such a swelling there is a circumscribed epithelial hyperplasia, but the elevation above the normal level of the skin is caused mainly by a localized swelling of the dermis.

Effects of Repeated Applications

When the skin of a group of mice belonging to the same strain is subjected to application of a 0.6-percent solution of methylcholanthrene in benzene repeated on alternate days, there is again a discrepancy in the skin reactions of different individual mice similar to the differences

observed after a single application. During the first week, the skin changes are similar in all the animals: a degeneration of the epidermal epithelium and the hair follicles in the central area, with a differentiation of the epidermal epithelium in the peripheral areas and the beginning of regenerative processes proceeding mainly from the hair follicles in the peripheral areas. Subsequently, however, the skin of individual mice reacts very differently to the continued application of the carcinogen. In some individuals the changes are rapidly progressive leading to the development of a carcinoma within 80 days or less. In another group the effect on the skin remains almost stationary after the second week when the process of regeneration has been completed, so that in this group the skin shows only slight changes at a time when the process of carcinogenesis has been completed in the first-mentioned group. In the bulk of the animals the skin shows changes intermediate between those seen in the two groups representing extreme differences. These individual differences in the histologically visible skin changes are reflected in the variations of the period of induction as shown in figure 1 of the preceding paper. It may be pointed out once more that under our experimental conditions eventually all the mice react to repeated applications of the carcinogen by developing a skin carcinoma.

Following is an account of the skin changes seen in the animals reacting rapidly to the carcinogen. They are illustrated in plate 4 where they are arranged according to the increasing number of applications of the carcinogen, which implies a corresponding increase in the time after the first application as in plate 3, which serves as a comparison. Since the applications of methylcholanthrene are repeated on alternate days, one would expect to find an intensification of the changes observed after a single application, namely, a progressively increasing destructive effect in the central area with a rapidly progressive hyperplasia in the peripheral areas. But this is not what happens. In fact, many of the changes induced in the skin during the first 2 or 3 weeks do not give the impression of being additive effects. The regeneration of epithelium in the central area is delayed but not prevented. Ulcerations do not occur at that early stage as a rule; and if they do develop, they are very small. In the peripheral areas the epithelial hyperplasia differs from that induced by a single application in a nonrefractory skin, not so much by the degree of hyperplasia in the epidermal epithelium as by showing more pronounced irregularities in the arrangement of the various cell layers and more pronounced differences between the degree of enlargement of individual cells, some of which become giant cells. Mitotic activity in the epidermal epithelium is not noticeably greater than after a single application of the carcinogen to a nonrefractory skin after the same interval.

There are, however, changes which do give an impression of being additive. The hyperkeratosis is more massive after repeated application. The altered hair follicles in the peripheral areas show an additive effect more clearly than the epidermal epithelium (pl. 4, *D*). They become greatly elongated, and broadened and dividing cells are seen more frequently than in the epidermal epithelium. Even the unaltered and still functioning hair follicles outside the peripheral areas often show an extraordinarily high degree of mitotic activity. The keratinized cysts, when present, show considerable enlargement owing to an increase in their contents. The changes in the dermis, the vascular dilatation and the edematous swellings, are

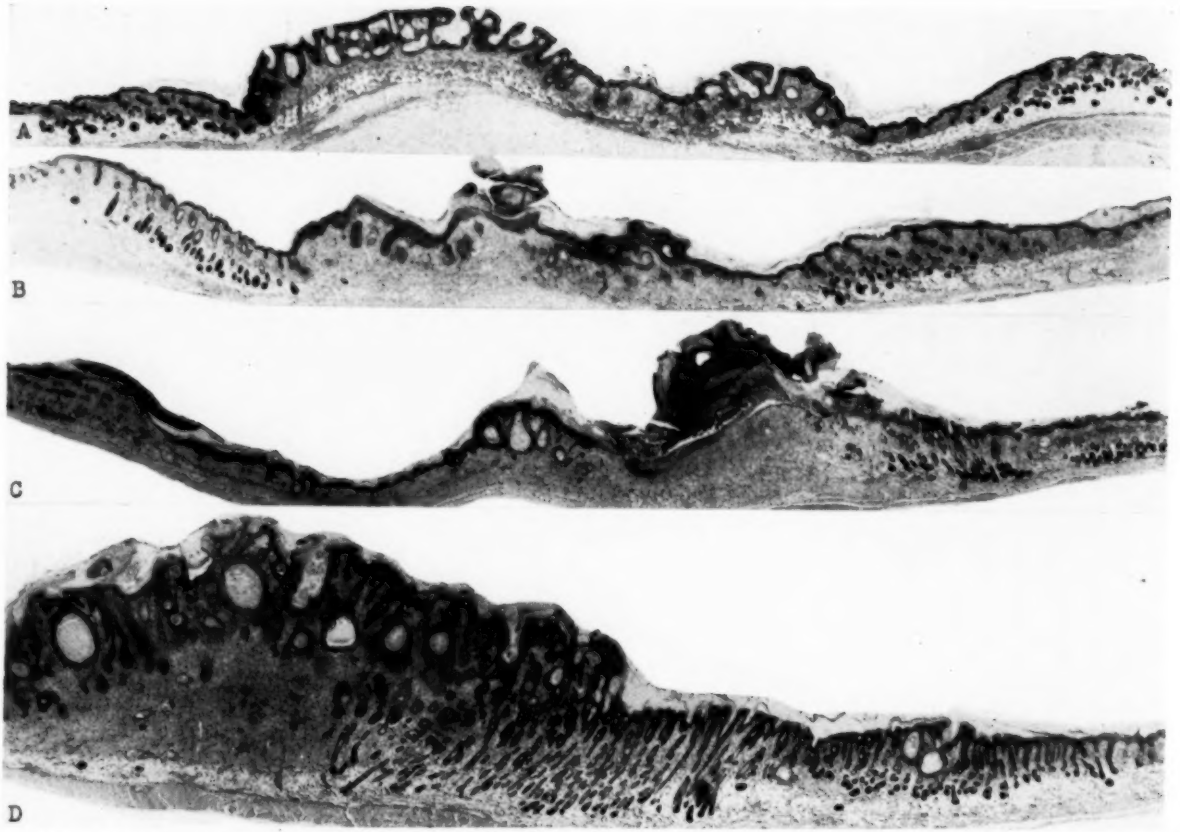

PLATE 4.—Mouse skin after repeated applications of a 0.6-percent solution of methylcholanthrene in benzene. *A*, 6 times in 14 days; *B*, 8 times in 18 days; *C*, 10 times in 23 days; and *D*, 12 times in 26 days. Transverse sections. × 13.

also greatly intensified by the repeated applications. It should be borne in mind, in making this comparison between the two experimental series, that a persistent epithelial hyperplasia occurs sooner or later in all the animals subjected to repeated applications of the carcinogen but is found in only a fraction of the mice treated with a single application of the carcinogen.

During this preliminary period, which covers a minimum of from 2 to 3 weeks but may extend to 10 weeks, the reactive hyperplasia induced initially by the carcinogen seems to be held back by the toxic effects of the carcinogen on the epithelium. When this period has passed, the skin behaves as if it had adapted itself to, or protected itself against the toxic effects of the carcinogen, and it then reacts to a subsequent application with a more active epithelial proliferation associated with frequent mitoses both in the epidermal epithelium and in the altered hair follicles. This increased mitotic activity usually affects groups of epithelial cells lying close together. The lining of the keratinized cysts also proliferates and increases in thickness. This leads to a massive and very irregular hyperplasia which may present a picture such as that illustrated in plate 4, *D*. In its general appearance it closely resembles a condition occurring in man, the epithelioma adenoides cysticum of dermatologists, and recognized there as a precancerous condition. If exposure to the carcinogen continues, the thickened epithelial lining of the keratinized cysts proliferates further. This leads to the development of a broad-based heavily keratinized papilloma. The malignant transformation of the epithelium begins, as a rule, in a circumscribed area of the hyperplastic epithelium and not necessarily throughout the whole papilloma. The statement that a papilloma is "transformed" into a carcinoma is incorrect and misleading. A carcinoma may also develop from the hyperplastic epidermis lying outside a papilloma in its immediate neighborhood. Figures illustrating this relationship have been published in a paper by Cramer (*4, p. 551*). A carcinoma may also appear in an area of hyperplastic epithelium in which there has been no preceding papilloma. This is illustrated in figure 2.

The reaction of the skin epithelium to repeated applications of methylcholanthrene is, therefore, not one which progresses continuously but rather one which is broken up into different phases. This conclusion agrees with the results of observations carried out in this laboratory by Cooper and Reller (*5*) as part of this group investigation. They counted the number of mitoses in the epithelium of the ears treated with methylcholanthrene and found an initial increase of mitotic activity followed by an inhibition, which, in turn, gave way to a second and more pronounced increase in the number of cell divisions.

The length of the various phases preceding carcinogenesis cannot be given in actual figures, since, as already stated, the skin of different individuals, even when they belong to an inbred strain of mice, does not react equally readily to the carcinogen. As the specimens selected for illustration were taken from those mice which reacted most rapidly to the carcinogen, the time indicated in the description applies only to the individual specimen, and it must not be assumed that all the animals subjected to the same treatment will show the same phase at that time.

In those animals in which the development in the skin of a carcinoma occurred after a long period of induction, during which the skin had been subjected to the effect of a large number of frequently repeated applications of methylcholanthrene,

FIGURES 1 and 2.—Mouse skin painted three times weekly with a 0.6-percent solution of methylcholanthrene in benzene. Figure 1, epidermis only slightly hyperplastic after 17 paintings in 40 days. Figure 2, diversity of reaction of epidermis after 36 paintings during 84 days; with carcinoma invading dermis (at right), slightly hyperplastic epidermis, regenerating hair follicles, benign papilloma, and precancerous papilloma (at left). Axial sections. × 12.

the skin remained refractory to the action of the carcinogen over a remarkably long period (fig. 1). In such individuals the degree of hyperplasia in the epidermal epithelium was slight, even after a period long enough to have induced a carcinoma in susceptible mice, and the hair follicles in the peripheral areas were greatly elongated but retained their usual structure and apparently their function. What is even more surprising is that in such animals the skin changes, induced by the application of the carcinogen repeated for many weeks, were much less pronounced than those induced in a susceptible animal by a single application.

It should be noted that under the experimental conditions observed in this series, namely, a highly potent carcinogen in a relatively high concentration applied repeatedly to a large area for a long period, even a refractory skin can be transformed eventually into one in which cancer will develop. This is illustrated in figure 2. This specimen was selected because it combines a number of interesting features. It is an axial section of skin which had received within 84 days 36 applications of a 0.6 percent methylcholanthrene solution. The main part of the section shows a resistant condition of the skin very similar to that represented in figure 1, namely, a slight degree of epithelial hyperplasia with, in one part, considerable elongation of the hair follicles. The left end of the section is occupied by two papillomas showing as yet no signs of a development of malignancy. These two benign tumors are of two different types: one is a sessile papilloma, the other a broad-based papilloma. Sessile papillomas are not as a rule precursors of a malignant development in their neighborhood. On the contrary, they have a tendency to disappear when the tissue constituting the stalk atrophies, presumably as the result of local circulatory disturbances. The broad-based papillomas, on the other hand, frequently precede the development of a carcinoma in their immediate neighborhood and may, therefore, be called the precancerous type. At the other extremity of the section there is a fully developed early carcinoma arising from a small area of strongly hyperplastic epithelium, which did not manifest itself by a papillomatous excrescence. In fact, to naked-eye inspection this area did not indicate the presence of an abnormal condition, but palpation revealed a sharply circumscribed thickening of the skin about 3–4 mm. in diameter. The occurrence of four such different conditions in an area of skin which had been subjected to the same treatment is an impressive illustration of the localization in the skin of the factors mainly concerned in the development of skin cancer. One curious finding in our various series was that on a few occasions sarcomas developed instead of carcinomas and that most of these neoplasms appeared in animals in which the skin epithelium showed only a slight or moderate degree of hyperplasia and was, therefore, refractory to the carcinogen. Moreover, in a few animals the sarcomas did not appear in or even near the central area as the carcinomas did, but quite outside it.

Ulcerations sometimes appear in the skin area exposed to the action of the carcinogen, but not in the first week, when in view of the primary destructive effect one might expect them to develop. They may appear, before a carcinoma or a papilloma has developed, as shallow defects which frequently heal over very rapidly, while at other times they persist for some time. Their appearance is irregular, and their relationship to carcinogenesis is obscure. It can be stated, however, that most carcinomas develop without a previous ulceration. Occasionally an ulcer may appear outside the skin area exposed to the

carcinogen. These are most likely the result of the animal's scratching itself. This explanation may account also for some ulcerations on the midline of the back in its anterior portion which the animal can reach with its hind leg, but not for ulceration in the posterior part of the midline. The appearance of ulcerations as a result of a fully developed carcinoma breaking through the skin needs no explanation.

In the dermis, the outstanding change produced by the repeated application of a benzene solution of the carcinogen is a progressively increasing dilatation of the blood vessels which runs parallel with the carcinogenic response of the skin, so that in the refractory skin it is much less pronounced than in the skin showing a rapid hyperplastic reaction. The dermis shows, in addition, an infiltration with macrophages and other wandering cells. One of the functions of the macrophages is concerned with the destruction of the hair follicles in the central area. In the later stages accumulations of mast cells are seen frequently, especially in the neighborhood of areas of massive epithelial hyperplasia; but as they are not a regular occurrence and as the normal function of mast cells is unknown, it is difficult to interpret the significance of this phenomenon.

The changes seen in the various stages of carcinogenesis for reasons of convenience may be considered as proceeding in three phases: (1) Traumatization and repair; (2) excessive repair leading to epithelial hyperplasia; (3) factors determining the development of skin cancer in a hyperplastic skin area.

TRAUMATIZATION AND REPAIR

The first effect, namely, the transformation of the normal epidermal epithelium consisting of two layers of small, undifferentiated cells into an epidermis consisting of five or six layers of large differentiated cells is not specific for the carcinogen. It can be induced by various kinds of local traumas inflicted on the epidermis. Thus painting with benzene alone produces this effect, but this is not followed by the breaking down of the epidermal epithelium in the central area. The destructive effect is due to the carcinogen. The use of benzene as a solvent for the carcinogen involves, therefore, a slight additional trauma which by itself is noncarcinogenic. The immediate effect of a single application of methylcholanthrene on the epithelium of the epidermis and of the hair follicles is a toxic one. With the strain of mice used in our experiments the immediate reaction in different individuals to a single application of a 0.6 percent benzene solution of the carcinogen is fairly uniform.

Reference to an initial toxic and destructive effect of a carcinogen on the skin epithelium is made by Wolbach (*6*, *7*) and by Page (*8*). Further evidence is found in observations by Shear (*9*) in his experiments on the experimental production in mice of sarcomas by the subcutaneous injection of pure chemical carcinogens. Animals injected with potent carcinogens, of which methylcholanthrene is one, developed ulcerations at the site of injections. Shear goes on to say ulceration—

> has been so regular an occurrence that if a newly synthesized polycyclic compound produces ulceration at the injection site in the first few months, it has come to be expected that tumors [sarcomas] will usually develop shortly thereafter (*9*, *p. 527*).

More recently Pullinger (*10*) has described in some detail the earliest changes in the mouse skin appearing after a single application to a small skin area of methylcholanthrene and some other potent carcinogens in a 0.3-percent solution in acetone. She records a progressive swelling of cells, nuclei, and nucleoli, with cytoplasmic degeneration which occa-

sionally leads to cell death. There is also an increase in the number of cells with evidence of differentiation. The effect is summarized as "severe damage to epithelium and acute inflammation of the subepidermal tissue" from which the cells recover. The changes induced by potent carcinogens are considered to be specific and different from the damage inflicted by such chemical substances as benzene, chloroform, and cantharidin.

A toxic destructive effect on connective tissue cells of the mouse has also been observed in experiments designed to induce sarcomas by the subcutaneous implantation of pellets containing small amounts of methylcholanthrene or other potent carcinogens. Wolbach, also Hval (*11*) and Orr (*12*) have recorded such effects, while Peacock and Beck (*13*) have pointed out that the sarcomas originate some distance away from the site of the carcinogen. The only discordant observations are those of Dunning, Curtis, and Eisen (*14*), who worked with rats. They hold that the development of malignancy depends on the direct contact between the carcinogen and the cell but agree that the effect of the carcinogen on the cell is an injury. It is probable that this discrepancy is due to differences in dosage. Shear (*9*) has pointed out that the ulceration of the skin which developed in his injection experiments could be avoided by diminishing the dosage but that sarcomas could still be induced, although less rapidly and less frequently. As has been pointed out in this paper, skin carcinomas also can be induced in the absence of preceding ulcerations. In fact, the appearance of ulcerations or of other less obvious forms of cell death is merely a convincing demonstration of the presence of a substance having a toxic, injurious effect on cells, which would not be recognized so clearly by dosages small enough to stop short of death of cells.

Evidence of a more general nature on the toxic action of carcinogens on living organisms has been supplied by the work of Haddow (*15–18*) and his collaborators. They have shown that almost all carcinogenic hydrocarbons have a toxic effect both on the normal organism and on malignant tumors growing in an aninal. This has been confirmed by Lees (*19–21*). The toxic effect manifests itself by a retardation of growth of both the normal animal and of any malignant tumor the animal may be carrying. Another manifestation of the toxic effect is the failure to breed from females subjected to the repeated application of chemical carcinogens. This is seen in many, but not in all, strains of mice. A toxic effect of chemical carcinogens on cells has also been demonstrated in observations on tissue cultures (Earle and Voegtlin (*22*)).

The point we wish to make may perhaps be made clearer by a comparison of the action of methylcholanthrene with that of Roentgen and radium rays. Such a comparison is justified, since these physical agents are also carcinogenic if given in small doses at suitable intervals. They resemble methylcholanthrene also with respect to their other biologic effects. Large doses of these rays induce a marked and obvious destructive effect on the skin. If the dose is diminished, their toxic, injurious action manifests itself by epilation and vasodilation; and if an attempt is made to define more clearly their effect on living cells by studying it in tissue culture, an elaborate investigation is required to establish the fact that they inhibit cell division. When these rays are applied not locally but to the animal organism as a whole, they resemble methylcholanthrene and other chemical carcinogens in producing an ar-

rest of growth. In this connection, it is interesting to note that Orr (*23*) from a study of the changes in carcinogenesis of the liver by butter yellow concludes that this dye has a destructive effect on the liver cells.

The destructive effect on the epidermal epithelium observed by us was obtained with a 0.6-percent solution of the carcinogen. We have not yet studied systematically the effects of the weaker concentrations usually used, where the traumatic effect may be less obvious. It is, therefore, important to recall that carcinogenesis is effected more rapidly and in a higher percentage of cases with a 0.6-percent solution of the carcinogen than with a 0.3-percent solution, other experimental conditions being equal.

Regeneration of Skin in Response to a Physical Trauma

Since methylcholanthrene induces a destruction of the epidermis, it is interesting to compare the immediate effect of a single application of a carcinogen with an epithelial destruction by a simple mechanical or physical trauma such as burning. This also induces the process of differentiation in the adjoining epidermal epithelium. When the narrow tip of a heated glass rod is drawn lightly along the middle line of a mouse's back, the histologic examination of the skin 1 or 2 days later shows a central area of destruction flanked on either side by a differentiated epidermal epithelium of several layers. This differentiated epithelium shows minor differences from the change appearing after methylcholanthrene has been applied.

After a physical trauma such as that induced by a hot glass rod, the arrangement of the epithelial layers is more regular and the cells in each of the different layers are more uniform in size and appearance than after methylcholanthrene. There is the further similarity between the effect of applying methylcholanthrene and a physical trauma such as heat that in both conditions the immediate effect of cell destruction is followed by a similar process of regeneration (fig. 3). The differentiated epithelial cells of the epidermis at the edges of the area where cells have been destroyed or undergone degeneration become elongated and spread over this area as the result of active cell division, which takes place both in the epidermal epithelium and also in the hair follicles underneath the differentiated epithelium.

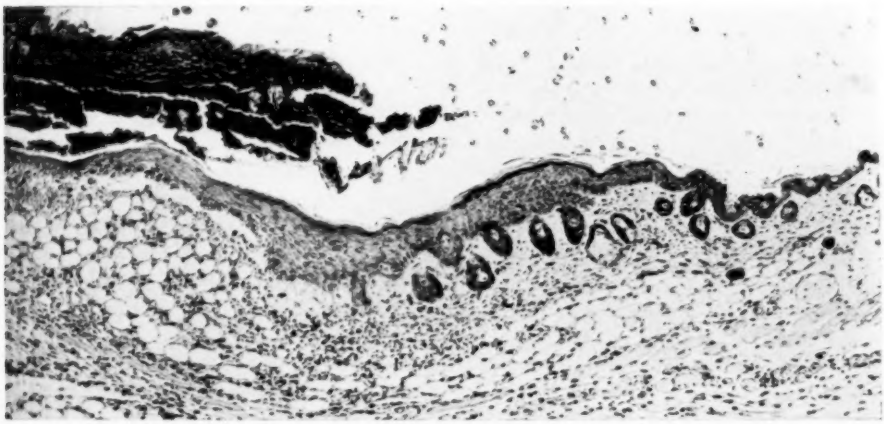

Figure 3.—Healing wound in mouse skin 3 days after a burn, showing differentiated epidermis. × 70.

In this regeneration the epithelial cells of the hair follicles play a more important part than has been generally recognized, for the colchicine technique shows dividing cells in the hair follicles in extraordinarily large numbers. This contribution of the epithelium of the hair follicles to the regeneration of the epidermal epithelium resembles the process of epithelial regeneration in the small intestine brought about by active cell division in the crypts of Lieberkühn.

Here the similarity between the two conditions ends, and there are significant differences. A simple mechanical trauma such as a burn or a cut produces its effect immediately, while the chemical trauma produced by a chemical carcinogen takes several days to develop, resembling in that respect such physical carcinogenic traumas as those induced by ultraviolet light, Roentgen rays, or radium rays, and indicating a more complex process. After the chemical trauma induced by a carcinogen, the process of epithelial repair is more dependent on the mitotic activity of the epithelium of the hair follicles than of the epidermal epithelium, where mitoses are seen much less frequently than in the hair follicles. After a single mechanical trauma the process of regeneration is arrested when the integrity of the epidermal epithelium has been restored, and this restored epithelium reacts to subsequent applications of a mechanical trauma in essentially the same way.

The chemical traumatization by a single application of methylcholanthrene is able to induce in a considerable proportion of the animals a process of excessive regeneration. This regeneration continues progressively for a long period after the defect has been repaired and leads to a massive epithelial hyperplasia which does not, as a rule, progress to the development of cancer unless the application of the carcinogen is repeated several times. In the majority of animals, however, the skin is refractory to a single application of the carcinogen in the sense that a single application of the carcinogen is not followed by a process of excessive regeneration but by what appears to be merely a restoration of the epithelium. But the reaction of this restored epithelium to subsequent application of the carcinogen has changed. It has become more resistant to the traumatizing effect and more apt to respond to repeated applications of the carcinogen by an excessive regeneration. As a result, even the most refractory skin can, by repeated applications of a sufficiently powerful chemical carcinogen, be induced to undergo an epithelial hyperplasia culminating in cancer, while it has not as yet been possible to bring about this result experimentally by repeated application of a mechanical trauma. The process of excessive regeneration whether induced by a single or by a repeated chemical traumatization by methylcholanthrene constitutes the second phase.

EXCESSIVE REGENERATION LEADING TO EPITHELIAL HYPERPLASIA

A systematic study of the effects produced in the skin during the first 4 weeks following a single application of methylcholanthrene has revealed the fact that in a proportion of mice, which in the Swiss strain we estimate to be about 25 percent, the skin epithelium responds with a progressive hyperplasia involving both the epidermal epithelium and the epithelial cells of the hair follicles, which have undergone morphologic changes accompanied by cessation of their normal function of forming hair. The hyperplasias thus induced may reach a high degree and may even after a longer interval lead to the formation of a papilloma which persists

without, however, becoming malignant. The animals reacting in this way may be called susceptible mice.

The remaining 75 percent of the animals do not respond to a single application of the carcinogen by a markedly excessive process of repair. A similar systematic study of the effects of rapidly repeated applications of the carcinogen over a longer period of 8 weeks has revealed the equally significant fact that of the 75 percent about one-third remain for a long period so highly refractory to even frequently repeated applications of the carcinogen that the skin epithelium fails to respond by a progressive epithelial hyperplasia of either the epidermis or of the hair follicles. As a result, the skin of a group of mice, belonging to the same strain, kept under identical conditions, and treated with the same solution of methylcholanthrene, may show the apparently paradoxic result of a high degree of hyperplasia following a single application of the carcinogen in some animals, while other mice which have received rapidly repeated applications of the carcinogen over the same or even a much longer period show merely a somewhat irregularly differentiated epithelium without any marked degree of hyperplasia.

THE DEVELOPMENT OF CANCER IN A HYPERPLASTIC SKIN AREA

In experimental carcinogenesis of the skin, a carcinoma develops always in an area of skin in which the skin has undergone a considerable degree of hyperplasia. But although, as just pointed out, a considerable degree of hyperplasia and even the formation of a papilloma can be elicited by the single application of a strong carcinogenic stimulus to a susceptible skin, cancer does not develop as a rule. Carcinogenesis requires the repeated application of a carcinogenic stimulus, except in a very susceptible skin. This means that the papilloma or the massively hyperplastic epithelium produced by a single application are not conditions which carry in themselves all the factors necessary for the development of a carcinoma. A new event has to supervene for malignancy to develop in these conditions, and when that happens the new event is restricted to one, or at most a few, circumscribed centers within the hyperplastic epithelium.

The conclusion follows that a fundamental difference exists between a benign neoplasm and a malignant one. That the former is not merely a stage of transition to the latter is in agreement with the experience in human cancer. In man benign neoplasms do not as a rule undergo a change into malignant ones (Ewing (*24*)). Observations on neoplasms in animals lead to the same conclusion. In rats, for instance, the mamma is frequently the site of benign neoplasms. These benign mammary tumors in rats have been studied in great detail by Heiman (*25*), who has transplanted them and followed their behavior throughout successive transplantations, when they always retain their benign neoplastic character and do not become transformed into carcinomas.

Carcinogenesis in the skin is, therefore, a discontinuous process. In the experimental skin carcinogenesis as studied in mice, it proceeds in a series of stages described in the three phases mentioned above. In the susceptible skin the second phase follows automatically on the first; in the resistant skin the second phase has to be elicited by repeated application of the carcinogen.

THE FACTORS DETERMINING SUSCEPTIBILITY

A 0.6-percent solution of methylcholanthrene is one of the most powerful carcinogenic stimuli known, as evidenced by the fact that on suitable application it will induce cancer not only in susceptible but

also in resistant mice in 100 percent of the animals. With a weaker carcinogenic stimulus, the experimental development of cancer is restricted to the susceptible individuals. The spontaneous development of cancer in man and in animals, which we may assume to be the result of weak carcinogenic stimuli in the great majority of individuals, is, therefore, dependent as much on the factors of susceptibility and resistance as on the presence of the carcinogenic stimulus. To call these differences "susceptibility" and "resistance" is not an explanation but merely a conveniently vague terminology which was introduced when the discovery of experimental carcinogenesis disclosed the existence of factors inherent in the organism which were necessary for the carcinogenic stimulus to be effective. Such factors of resistance and susceptibility to the development of cancer exist not only for the skin but also for other organs, and for some organs such as the mamma they can be concentrated by inbreeding. It has been shown that the susceptibility to cancer of one organ does not imply susceptibility to cancer of another organ. These factors, the nature of which is as yet very obscure but which are as important for the onset of malignancy as exposure to a carcinogenic stimulus, must therefore be analyzed separately for each organ.

In the skin, resistance or refractoriness and susceptibility are not concerned with the first phase of the reaction of the skin to the carcinogen, for in this phase there is very little difference between individual animals. They all show the traumatic effect fairly equally. The difference begins with the difference in the reaction following this traumatic effect, as described in the preceding section on the second phase of the action of methylcholanthrene. The fact that this difference manifests itself after a single application of methylcholanthrene has simplified the problem so as to make it possible to obtain an insight into the factors determining resistance and susceptibility of the skin to a carcinogenic stimulus.

In the technique of experimental carcinogenesis in the skin as generally employed, the application of the carcinogen is repeated at short intervals every second or third day. This technique is based on the assumptions that the epithelial hyperplasia is due to a direct stimulating effect of the carcinogen and that a continuous exposure of the epithelium to the carcinogen affords the most favorable conditions for eliciting the epithelial hyperplasia. All observers are agreed that the carcinogen is removed fairly rapidly from the site to which it is applied and is being transformed by the organism into other chemical compounds which are noncarcinogenic. For the skin, Beck and Peacock (*26*) have shown that the carcinogen disappears from the skin after about 4 days. They argue from this that an application of the carcinogen twice a week insures a continuous exposure of the skin to the carcinogen.

If this conception of the mode of action of skin carcinogens is correct, resistance and susceptibility can be explained by postulating for the resistant skin a more rapid removal, and for the sensitive skin a delayed removal of the carcinogen from the skin. Applied to our experimental conditions, this would mean that in the resistant mice the methylcholanthrene had been removed in less than 2 days, since our applications were made every second day, while in the susceptible skin it remained over a period of at least 4 weeks and even longer in those animals in which a single application was sufficient to elicit a carcinoma after 4–6 months.

If the continuous exposure is not an essential condition for carcinogenesis—and an experiment to be mentioned presently

shows that it is not—then the explanation just given fails to account satisfactorily for the fact that an application of methylcholanthrene repeated 20 times or more should fail to elicit a hyperplasia in the skin of some animals. An alternative explanation is that the direct action of the carcinogen on the normal epithelium is restricted to the production of a chemical trauma. Like other traumas this elicits automatically a reaction of repair, which is arrested when restoration has been accomplished. In other words, the reaction seen in the resistant skin to a single application of the carcinogen represents the normal reaction of the skin to the carcinogen. If that is so, the hyperplastic reaction to a single application seen in the susceptible skin may be accounted for by postulating an additional factor, the formation in the skin, in response to the action of the hydrocarbon, of a substance which stimulates the epithelial cells to excessive proliferation.

In order to test the validity of the conception that the excessive epithelial proliferation which leads up to the development of malignancy in the skin is elicited not by the direct action of the carcinogen, which has a toxic inhibitory effect, but by a substance formed in the skin in response to the carcinogen, an experiment was carried out in which methylcholanthrene was applied to the skin at long intervals (Cramer and Stowell (*2*)). In three experimental series of mice the same solution of the carcinogen was painted on skin areas of equal size at intervals of 2, 3, and 4 weeks, respectively. It was found that the amount of carcinogen, as indicated by the number of applications, necessary to elicit carcinogenesis by this method was much smaller than the amount required to induce carcinogenesis by the frequent application of the carcinogen repeated on alternate days. Moreover, in the three series treated by the discontinuous method of exposure the amount of the carcinogen necessary to induce carcinogenesis diminished as the interval between successive applications was prolonged. Such a result cannot easily be reconciled with the conception of a direct stimulating effect of the carcinogen of the epithelial cells.

One other fact, recorded previously (*1*), may be mentioned here, which is accounted for satisfactorily by this hypothesis, namely, the fact that the area of tissue subjected to the action of the carcinogen determines the rate of the process of carcinogenesis to a greater extent than was hitherto supposed. An increase in the skin area exposed to the carcinogen will increase the amount of the substance liberated by the skin.

CONDITIONS DETERMINING CARCINOGENESIS

When a skin area is treated repeatedly with a carcinogen at short intervals, the epithelial cells are subjected, as a result of the formation of this substance, to two antagonistic actions—the toxic action of the carcinogen and the stimulating action of the substance formed by the tissue in response to the carcinogen. The condition resulting from this combination may be summed up as "a process of inhibited excessive regeneration." In the susceptible animals, this process leads at once to an excessive regeneration; in the resistant ones, repeated applications are required to induce that stage. Cancer develops subsequently in one or more circumscribed centers in the area of excessively regenerated epithelium. Although the exact nature of the intracellular change which constitutes the transformation of a nonmalignant into a malignant cell is not known, most workers on cancer are agreed that this change affects the nucleus. Some describe it as a mutation, others as a change in the gene complex, and yet a third group postulates the

existence in the nucleus of an abnormal substance which may either be formed by the cell and possess genelike functions, as Stanley suggests, or may lead an independent biologic existence in the normal organism as a nonpathogenic virus until it is enabled to enter the nucleus of cells placed under abnormal condition by treatment with carcinogens.

There is considerable cytologic evidence to support the view that conditions involving a combination of stimulation and of inhibition of mitotic activity may lead to nuclear changes. This evidence was set forth very clearly 25 years ago by Boveri (*27*) who, basing his conclusions on a lifelong study of the factors determining abnormal cell divisions in invertebrate cells and without having made a direct study of cancer cells, attributed the transformation of a normal into a malignant cell in vertebrates to just such a combination. Since the conception that a carcinoma results when cells are subjected to several sets of conditions, one group of which stimulates the cells to increased mitotic activity while the other group inhibits it, is based on a study of carcinogenesis in the skin, it is necessary to consider briefly whether it can be extended to other epithelial tissues.

Methylcholanthrene can induce cancer in the mamma (Strong and Smith (*28*), Dunning, Curtis, and Eisen (*14*)), in the stomach and in the small intestine (Lorenz and Stewart (*29*, *30*)), and there is no reason to suppose that its mode of action on these tissues differs from that on the skin. The liver does not respond to this carcinogen by the development of a primary cancer (Shear, Stewart, and Seligman (*31*)). But for the liver it has been shown that the aniline dyes which are carcinogenic for this organ have a toxic destructive effect on the liver cells (Wolbach (*6*, *7*), Orr (*23*), Emmart (*32*)). There are, thus, a considerable number of tissues in which the carcinogenic effect of a chemical compound is associated with a primary toxic effect on the epithelium. There is also the carcinogenic effect of such physical agents as Roentgen rays, radium rays, and ultraviolet rays for which it has hitherto been difficult to establish an analogy with the mode of action of chemical carcinogens. The conception outlined herein makes it possible to establish such an analogy.

On the other hand, the development of cancer in the mamma in response to estrogenic hormones cannot be attributed to a toxic injurious effect of the hormones on the mammary epithelium. The physiologic function of the estrogenic hormones is to induce a proliferation of the mammary epithelium. But the observations of Bittner on inbred strains of mice have shown that the estrogenic hormones by themselves are not able to induce mammary cancer; the action of a second substance present in the mamma of high-cancer strains and secreted in the milk is required, the so-called milk factor. Since cancer can be induced in the mamma also by the local application of methylcholanthrene, one would either have to assume that there are two different types of carcinogenesis for the mamma of the mouse, or, if the two types are built on the same pattern of subjecting cells to a substance stimulating mitotic activity and to another inhibiting it, one would have to postulate for the milk factor the property of inhibiting proliferation of the mammary epithelium.

In this connection it is interesting to note that after this paper had been sent in for publication, Strong and Williams recorded the observation that mammary cancer can be induced by methylcholanthrene in mice belonging to strains highly refractory to the spontaneous development of mammary cancer (Cancer Research, 1: 886–

890 (1941)). Such animals are also very resistant to the carcinogenic effect of estrogens.

SUMMARY AND CONCLUSIONS

The histologic changes taking place in the mouse skin in the first 2 months after the application of methylcholanthrene to a large skin area were studied in 94 mice of the Swiss inbred strain. A standard technique of applying the carcinogen giving a near optimal carcinogenic response was used, and comparable parts of the skin were taken for microscopic examination. The effects of a single application and of repeated applications of the carcinogen were compared in the skin of animals killed every second day. Special attention was paid to the earliest changes immediately following the first application of methylcholanthrene.

The outstanding conclusion is that the immediate effect of the first application of methylcholanthrene is an injury to the skin epithelium. In subsequent applications of the carcinogen, the toxic effect on the epithelium was masked by an increasingly active stimulation of cellular proliferation. This altered reaction of the skin epithelium to the carcinogen—a process in which different individual animals showed strikingly great differences described by the terms "resistance" and "susceptibility"—can be accounted for adequately by postulating the formation in the skin of a substance stimulating the epithelium to excessive proliferation.

On this reasoning, the carcinogenic property of a hydrocarbon for the skin depends on its ability to inflict on the tissue an injury of a specific kind which leads to the formation by the skin of a substance inducing epithelial proliferation. The descriptive terms "resistance" and "susceptibility" are then explained as differences in the readiness with which the substance inducing epithelial proliferation is formed by the skin in response to the effect of the carcinogen. In the so-called susceptible mice this substance is formed readily even after a single application of the carcinogen; in the resistant mice numerous applications of a powerful carcinogenic stimulus are required, while weak carcinogenic stimuli fail to elicit its formation and to elicit the development of cancer. As a result, a susceptible skin will respond to a single application of the carcinogen with a massive epithelial hyperplasia within a few weeks, while frequently repeated applications will fail to do so in a resistant skin.

The validity of this interpretation has been tested experimentally by applying the carcinogen at long intervals of 2, 3, or 4 weeks, thus allowing the stimulating effect of the proliferins to act over a longer period without being diminished by the inhibitory toxic effect of the carcinogen. The results are in accord with the interpretation given. They show that the dose of methylcholanthrene necessary to induce skin cancer diminishes as the interval between the application of the carcinogen is prolonged.

Conditions favorable to the development of a carcinoma exist when epithelial cells are subjected to two opposing influences: one which stimulates mitotic activity and one which inhibits it. The implications of this concept are discussed.

The importance attributable, on the basis of our observations, to the toxic effects of the carcinogenic hydrocarbons makes it possible to understand the spontaneous development of cancer in man under conditions for which it is at present difficult to find an adequate explanation on the basis of the prevalent conception that a carcinogen has a direct stimulating effect on cellular proliferation. There is the fact, for instance, that such physical agents as

Roentgen rays and radium rays, which have a destructive action on cells and to which the skin epithelium is particularly susceptible, can induce cancer of the skin if given repeatedly in small doses. Another example is the spontaneous development of cancer in the stomach and in the esophagus where it may occur on the basis of a longstanding atrophic condition of the epithelium instead of an epithelial hyperplasia, as it does in experimental carcinogenesis. In these conditions an increased mitotic activity in the neighboring epithelium is induced in an attempt to compensate for the defect, while the conditions responsible for the atrophy persist and account for the inhibiting factor. While this conception of the etiology of skin cancer in response to a chemical carcinogen accounts adequately for the etiology of some forms of human cancer of the skin and of other organs, the development of malignancy need not necessarily always follow the same trail. This is discussed with reference to carcinogenesis in the mamma by methylcholanthrene on the one hand and by estrogenic hormones and the milk factor on the other.

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